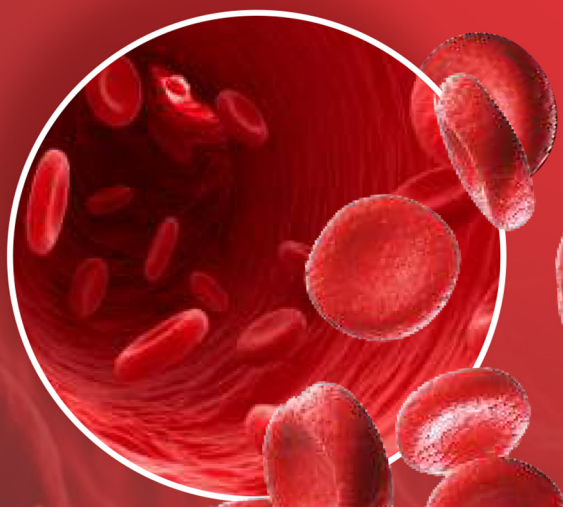
Seventh Edition

Hematology

for Students and Practitioners

Including Practical Hematology



Ramnik Sood

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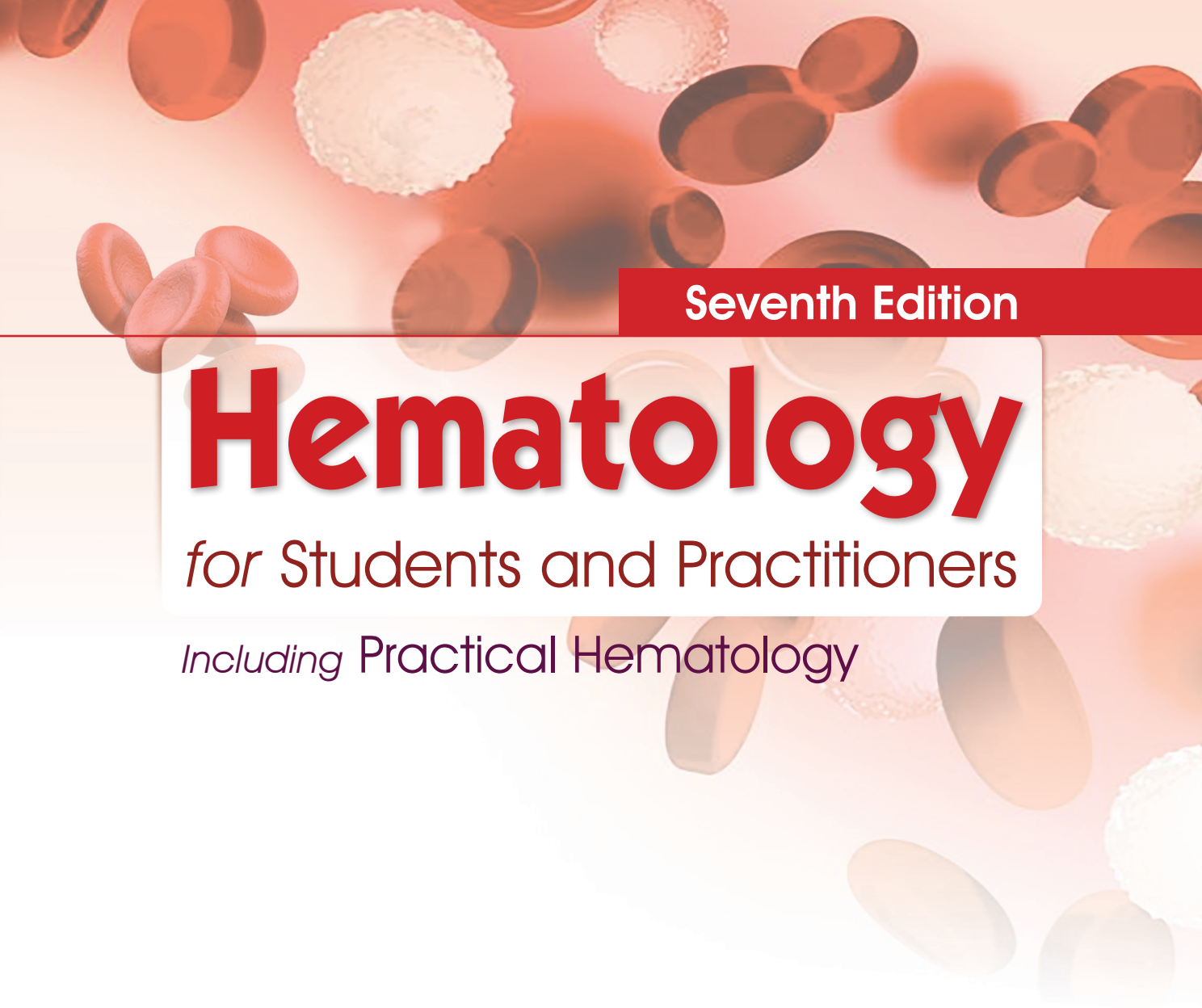

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Ramnik Sood
MBBS, PGEn&OH, MD (Path) Gold Medalist
Director, Speciality Diagnostic Labs, Panaji, Goa
Consultant 'A', UAE
Former Senior Visiting Consultant
Manipal Health Systems
(Manipal Group)
Bengaluru, Karnataka

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Corporate Office: 204 FIE, Industrial Area, Patparganj, New Delhi-110092
Ph: +91-11-49344934; Fax: +91-11-49344935; Website: www.cbspd.com; www.eduport-global.com;
E-mail: eresources@cbspd.com; marketing@eduport-global.com

Head Office: CBS PLAZA, 4819/XI Prahlad Street, 24 Ansari Road, Daryaganj, New Delhi-110002, India.
Ph: +91-11-23289259, 23266861, 23266867; Fax: 011-23243014; Website: www.cbspd.com;
E-mail: publishing@cbspd.com; eduportglobal@gmail.com.

Branches

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  Ph: +91-22-24902340 - 41; Fax: +91-22-24902342; E-mail: mumbai@cbspd.com
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to
my father

Preface to the Seventh Edition

The art and science of studying and diagnosing problems related to the red liquid connective tissue of our body has seen a sea change in the last century and about a quarter of this century. While importance and relevance of brain, heart, nerves, musculoskeletal system cannot be undermined, the logistical system of human body that carries nutrients, energy, micronutrients from the source of procurement/absorption to their processing organs and thereafter to every cell of the human body for eventual usage and subsequently gathering of wastes created to the end clearing and disposing organ of the body just cannot be overlooked. It is called blood. Blood which on the shirt or on the road signifies bad omen but the same flowing nicely through the vasculature signifies life. Stoppage of blood supply implies the ultimate end of our body and all that works within it.

In short, blood is the lifeline of a human body. It also assists in diagnosing most of the ailments that afflict to a human body and also serves as the vehicle for infusing lifesaving medicines too. It is also the army and navy of any living being, always ready to supply the fighting force—a line of defense in the microphages—better known as neutrophils. All antibodies are suspended in it too.

In the current century, automation has taken over to a great extent; however, unless one understands the basics and primary testing procedures, one cannot grasp what a machine is doing and how. So, tests of yesteryears are very much a part of this book as are random access walk away automated systems.

The book presents all three cell lines-related disorders and their diagnostic protocols. If a disease presents with any change in hematological parameters, you will find it suitably/amply covered within the covers of this book. Not forgotten are the clotting mechanisms, the blood banking line, nor has the hemoparasitology been ignored. The dread creating hemomalignancies are presented in desired details. Not much has changed in last decade in treating leukemias in any case.

Last three years, brought to the fore coronavirus disease (COVID), though still it is being discussed in journals in the form of article; however, basic accepted changes that blood undergoes have been lucidly introduced as the last chapter.

Not to forget the practical hematology that was always an integral part of the previous six editions always has been duly presented too.

So, if you are an undergraduate/postgraduate student or a technologist running a clinic-side laboratory—*This book is for you!*

From *alpha to zeta of hematology* is waiting to grab your kind attention, flip though the pages and enjoy an easily assimilable knowledge experience.

Happy reading !!!

Ramnik Sood

Preface to the First Edition

Medical book writing is basically an editing work. The voluminous textbooks are prepared from thousands of published articles, and the not so voluminous books (like the one you are holding) are prepared to provide 'longer acting (retainable) substance with a lesser dosage'. This book, though cheap cost-wise, is rich in its contents. Care has been taken not to miss any topic of use either in examinations or in practice. The systematic format and the presentation is easily palatable. Wherever necessary a list of normal relevant values has been given, the figures need no legends, as they are self-explanatory. I do claim that this book is more than sufficient for undergraduate theoretical and practical hematology. I hope that the red liquid blood presented here quite solidly in black and white would go a long way both for students and for the aware practitioners. Suggestions for improving the next edition would be gratefully accepted and appreciated.

Ramnik Sood

Contents

Preface to the Seventh Edition *vii*
Preface to the First Edition *ix*

1. History and Scope 1
2. Blood Formation 4
3. Red Blood Cells 36
4. Basic Principles in Diagnosing and Treating Anemias 65
5. Iron Deficiency Hypochromic Anemia 73
6. Megaloblastic, Macrocytic Anemias 96
7. Symptomatic Anemias 129
8. Hemolytic Anemias 158
9. Disorders of Hemoglobin Structure and Synthesis 250
10. Aplastic Anemia, Pancytopenia 293
11. White Blood Cells 312
12. Leukemias 333
13. Paraproteinemias 380
14. Lymphoid Tissue Tumors (Malignant Lymphomas) 397
15. Myeloproliferative Disorders 415
16. Platelets, Blood Coagulation, and Hemostasis 441
17. Coagulation Disorders 488
18. Thrombosis: Clinical Features and Management 538
19. Hypersplenism and Splenomegaly 558
20. Bone Marrow Transplantation 564
21. Immunodeficiency Disorders 568
22. Bone Marrow in Nonhemopoietic Disease 601
23. Hemoparasites 607
24. Practical Hematology 640
25. Immunohematology Blood Banking 722
26. COVID and Hematological Changes 766
27. Immunoassay: Instrumentation, Automation and Point-of-care Testing 769

Index *797*

CHAPTER

1

History and Scope

Presence of blood in human body has been known from those days when men used to inhabit forests and caves. However, the fact that blood circulates in our body was discovered by William Harvey a couple of centuries back. Many systems of medicine, Greek, Roman and Unani, etc. ascribe bodily diseases to impurities or abnormalities in the blood. It is a known fact today that most diseases—traumatic, inflammatory, infective, organic (not functional) and neoplastic (both benign and malignant) are accompanied by alterations in the blood. These alterations may be in the plasma, serum, blood cells, etc. Blood is being used extensively for diagnostic as well as therapeutic purposes. Maximum research has perhaps been carried out on this liquid connective tissue of our body. Always dynamic and moving; its stoppage leads to death of the tissue getting devoid of its blood supply.

Blood has been related to life and if one goes through the holy books—blood-bath or bloodshed or bloody war has been associated with death. The diagnosticians of yester years did not possess a microscope to see the simple changes that occurred in blood, all the same, they could see it grossly. They knew that if allowed to clot and settle in a transparent container, blood formed four distinct layers. At the bottom was found a dark red (almost-black, jelly-like) layer, above this was seen a red layer, still above was visualised a pale green or whitish layer. Atop all these were recognized a transparent, yellow serum. These layers were possibly related to the four humors, viz. black bile, sanguis, phlegm and yellow bile respectively. Health or disease was thought to be arising from a proper or improper mixture of these four humors. Even about 3 centuries back, physicians still believed the theory of humors. As we understand today, they did have a point—a Wintrobe's tube when centrifuged shows three layers—a bottom layer of packed red cells, a whitish creamy layer called the buffy coat and the topmost layer of plasma. One can obtain sufficient information just by carefully examining these layers. The fact is discussed in detail in the relevant chapters.

From seventeenth century AD till 1920, many individuals provided their hypotheses, theories and findings but the modern hematological concepts were provided first (in 1920) by Whipple, Minot and Castle. The last 50 years have seen tremendous advancement in the field of hematology, which now covers and embraces closely such diverse medical principles as biochemistry, cell biology, immunology, physical chemistry, molecular biology, genetics and nuclear medicine. Despite the complexities; Hb, TLC, DLC and ESR are perhaps the first investigations that are requisitioned by a physician or a surgeon. These are remarkably simple techniques and provide treasures of informations.

A perfectly normal person displays diurnal variations in Hb, TLC, DLC, etc. Changes occur in a pregnant woman. A cursory glance at the stained blood film may reveal the underlying cause of the complaints that the patient presents with. Burring of red cells

implies uremia, macro-ovalocytosis may point towards malabsorption or undernutrition, punctate basophilia or RBCs leads towards lead poisoning. Microcytic-hypochromic anemia with eosinophilia should make you think about a possible hookworm infestation. You name the disease or syndrome and blood shall show some sort of deviation from normal. How would you diagnose a hypochondriac? Before you refer the patient to a psychiatrist just do his ESR. A normal ESR should make you refer the case to a psychiatrist. Let me illustrate with an example—how can you differentiate true myocardial infarction from cardiac neuroses—the former shall show leucocytosis with neutrophilia with markedly raised erythrocyte sedimentation rate and the latter would show none of these findings. Indeed, these simple hematological investigations change the course of your thinking, diagnosis and hence the treatment. How does one assess the prognosis of organic disorders?—here again a simple investigation like ESR provides the much wanted invaluable information. If the ESR is gradually decreasing, the patient is on his way to recovery; on the contrary, if it is increasing, perhaps something else needs to be done for the patient is going on a downhill track. This is the diagnostic aspect, there is yet another facet, the therapeutic use of blood. You can choose whole blood or its fractions for various hematological disturbances. Are you going to do a prostatectomy or a thyroidectomy? Well, you will need blood to replenish the lost blood. The most frequently donated tissue is the red liquid connective tissue (blood), blood transfusion is a life-saving treatment in many instances. We are now doing bone marrow transplantation to help the victims of aplastic anemia and similar disorders where the bone marrow dries up. Simply said, *blood is life*.

A sound knowledge of clinical medicine is absolutely essential for any hematologist worth the name. The polluted environment of this disorganised modern era is leading to anemias, leukemias and other non-hematologic problems. A detailed history is a must with special reference to exposure to chemicals or drugs. Diet, menstrual history, history of gastrointestinal blood loss and any history of fever should also be inquired into. Family history with affliction related to a particular sex is also important. Take a close look at the skin, sclerae, tongue and nails, etc. Palpate the liver and spleen. Record any findings if obtained. Perform the investigations required at the earliest (i.e. soon after withdrawing the blood sample), delay can lead to erroneous results and consequent improper treatment. Although artificial intelligence of computers and machines is being utilised these days but nothing can replace the participation of human eye attached to a knowledgeable brain (the eyes cannot see what the brain does not know). A well-examined peripheral smear is a storehouse of information that can change the entire course of diagnosis and therefore treatment. No degree of automation can be compared to even a not so perfect hematologist. For instance, it is advisable to recheck the adequacy of platelets in a peripheral smear as blood cell counters can count dust particles as platelets. Similarly, cell counters count large stress lymphocytes and blasts in the same category, namely large lymphocytes and mistakingly they count late normoblasts as small lymphocytes. To say the least, do not overestimate the capability of cell counters while underestimating your own knowledge and judgement.

Nobody can deny the fact that *better diagnosis means better treatment and therefore quicker recovery and relief.* While reporting try and be descriptive—tell the physician what a schistocyte means and conveys. Just as a good hematologist may not know how to interpret an EEG, likewise, the ablest of physicians may have forgotten that stomatocytes can be seen in chronic liver disorders or burring or RBCs can be seen in uremia. Make suggestions to the physician for the ultimate benefit of the patient. For instance on performing ESR if

you see icteric plasma column—convey it to the physician, he may not have considered this at all.

The only malignancy where we can claim a cure rate is that of blood, a type of leukemia, the acute lymphoblastic leukemia. I cannot in any language or phrase express the full importance of blood in our body's system. One can go on endlessly, it is the horizon. One may think one has reached the end but just one look forward will convince you that you have just covered an anthill and not a mountain. This century shall make further revelation; revelations that we may have not dreamt about as yet. Can we teach our army, navy and airforce (the fighting system of our body, namely blood) to recognize tumors as foreign elements, if we can. Well, blood will show its loyalty and wage a war against the devilish tumor. The day is not very far when we will be able to do that and that shall be the most difficult battle won. It shall be a breathtaking event, a spectacle, a feat; man landing on moon will then be just a minor achievement.

CHAPTER

2

Blood Formation

Blood is a mesenchymal tissue and its components are:

1. Plasma
2. Red blood cells (erythrocytes, rubricytes)
3. White blood cells (leukocytes), and
4. Platelets.

In a normal healthy person there is a constant breakdown and fresh formation of various blood elements. However, minor daily physiological fluctuations do occur.

The erythrocytes transport the respiratory gases, oxygen and carbon dioxide, granulocytes and monocytes (leukocytes) are cells that can get out of the blood vessels and migrate among the cells of many tissues. These cells are important in inflammation and phagocytosis. Platelets are tiny, anucleate cells that contain molecules required for hemostasis, they also provide hemostasis through their ability to adhere, aggregate and provide a surface for coagulation reactions. Lymphocytes mediate highly specific immunity against microorganisms and other foreign particles/molecules. B lymphocytes confer immunity through production of specific soluble antibodies, whereas T lymphocytes direct a large variety immunity functions, including killing of cells that bear foreign molecules on their surface membranes. Inspite of such vast structural and functional differences, these cells are thought to be originating from a single type of cell. The process is termed as hematopoiesis. These processes of hematopoiesis include self renewal of stem cells, commitment of most progeny of stem cells to differentiate ultimately into a particular cell type, proliferation of the progenitor cells, and their differentiation along a pathway leading to a particular kind of mature blood cell. Absolutely essential for the process to continue, is the maintenance of a local environment conducive to survival of at least same portion of the cell lineage, production of necessary protein survival factors or growth factors.

SITES OF BLOOD FORMATION

During embryogenesis blood production sites change several times. Nucleated RBCs first appear in "blood islands" in the yolk sac (yolk sac hematopoiesis is dominant till 8 weeks of gestation). The yolk sac-derived RBCs do not lose their nuclei during maturation and they contain hemoglobin molecules of α and β chains. As soon as liver is organized, it becomes the major hematopoietic organ of the fetus during mid-gestation; the spleen also to a minor extent participates in the process. The RBCs produced by these organs are anucleate and the hemoglobin consists of α and fetal type β chains. Eventually, the bone

marrow takes over the process (and, it stays the exclusive post-natal site of hematopoiesis). During late fetal development RBCs begin to be produced that contain the adult type β chains.

Normal Sites

Fetus less than 2 months—yolk sac
From 2–7 months—liver, with minimal hematopoieses in spleen.
After 3 months—hematopoiesis starts in bone marrow.

Full term infant: Bone marrow is the only site for production of granulocytes, red blood cells and platelets. Production of lymphocytes and monocytes occurs mainly in the spleen. Lymph nodes and other lymphoid tissue, though liver and bone marrow produce these in much lesser number.

After birth: Same as above except that the monocytes are provided by, the bone marrow; spleen and lymphoid tissues contribute minimally.

The hematopoiesis can be subdivided into 3 stages:

a. Mesoblastic period
b. Hepatic period and
c. Myeloid period

Figure 2.1 pictorially indicates the sites of (and periods of) blood formation *in utero*.

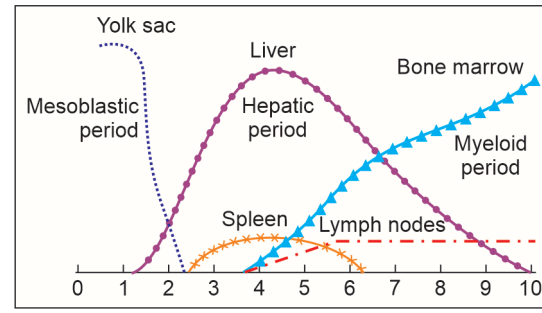


Fig. 2.1: Fetal hematopoiesis

Distribution of Active Bone Marrow in an Adult

The weight of active red bone marrow in an adult is about 1000 grams.

Site	*Percent of total*
Cranium and mandible	13
Humeri, scapulae, clavicles	8
Sternum and ribs	10
Vertebrae	28
Pelvic bones	34
Femur	4

With increasing age, peripheral skeleton produces less blood cells and the function is taken over by axial skeleton mainly. After the age of 4 years fat cells begin to appear in the long bones. Around age of 18 years, hematopoietic marrow is found in the sternum, ribs, pelvis, vertebrae and skull.

Abnormal Sites

Extramedullary hematopoiesis (myeloid metaplasia): In certain disorders the fetal hemopoietic organs revert to their old function supported by the reticulum cell which retains its potential hemopoietic activity. This occurs when bone marrow cannot fulfil the requirements or demand imposed upon it any further.

The spleen and liver are the most commonly encountered sites, but hematopoiesis in lymph nodes, and less commonly, adrenal glands, cartilage, broad ligament, thrombi, adipose tissue, intrathoracic areas, kidneys and endosteum also has been recorded. The presence of megakaryocytes in these areas differentiates the process from leukemic infiltrates.

Examples

- Erythroblastosis fetalis
- Pernicious anemia
- Thalassemic anemia
- Sickle cell anemia
- Hereditary spherocytosis
- Various leukemias
- Aplastic anemia (rarely)
- Myelosclerosis
- Secondary carcinoma of the bone.

DEVELOPMENT OF BLOOD CELLS

IN AN ADULT

Hematopoiesis occurs in the extravascular part of the red marrow. A single layer of epithelial cells separates the extravascular marrow compartment from the intravascular compartment (venous sinuses). When blood cells produced in the marrow are almost mature and ready to circulate in the peripheral blood, the cells leave the marrow parenchyma by passing through the fenestrations in endothelial cells and emerge into the venous sinuses.

The various cell lineages in bone marrow originate from the pluripotent hematopoietic stem cells. The pluripotential stem cell provides a cellular reserve for the stem cells committed to the lymphoid stem cell line for lymphopoiesis and to the nonlymphoid stem cell line for producing the other hematopoietic cells. The nonlymphoid (or myeloid) committed stem cell produces the erythrocytic, granulocytic (neutrophilic, eosinophilic, basophilic), monocytic macrophage, and megakaryocytic-platelet lines. The lymphoid committed stem cell produces lymphocytic and plasmocytic cells. The pluripotential stem cells are not as such identifiable in bone marrow smears, but when isolated in cell culture systems they have been observed to be small round cells with fine chromatin, nucleoli, and blue nongranular cytoplasm (akin to an early lymphoid cell).

Most of the knowledge gained as regards hematopoiesis has been derived from non-human mammalian experimental models, e.g. mice. The hematopoietic stem cell has been defined as a colony forming unit (CFU). In a classic experiment, Till and McCullock irradiated mice and then injected suspensions of marrow cells intravenously. About 10 days later, nodules of injected marrow could be observed on the surface of the spleen and in microscopic study of the splenic tissue. These nodules were named "spleen colonies," and the progenitor cells that formed these colonies were called colony-forming unit-spleen (CFU-S).

Under the influence of the splenic microenvironment, an injected stem cell from a suspension of marrow cells proliferates and differentiates into a larger colony. Enumeration

of the colonies allows for an indirect method of quantitation of the number of injected CFU-S. Each colony is derived from a single stem cell. Proof of the clonality of the nodules is substantiated by using cells with chromosome markers induced by radiation when 95% or more of the metaphases in the colonies are observed. In addition, if a colony is removed cautiously from the spleen two weeks after injection and a suspension of cells is made and injected into irradiated mice, numerous colonies are observed. Such evidence demonstrates the self-renewing capacity of CFU-S in the colonies.

Spleen colonies observed at first were primarily composed of erythroid cells with a few megakaryocytes and granulocytes, but later the colonies were noted to be mixtures of these cell times. Thus, the committed myeloid (pertaining to marrow) stem cell (CFU-S) is considered to be multipotent and to give rise to granulocytes, erythrocytes, monocytes, and megakaryocytes. Hence, the term CFU_{GEMM} (granulocytic, erythrocytic, myelocytic, monocytic) has been suggested and may be substituted for "CFU-S" (Fig. 2.2).

In vitro culture methods now provide another assay system for hematopoietic stem cells. Progenitor stem cells (CFU-S) have been identified by their activity to form large *in vitro* colonies on semisolid culture media which contains growth factor or colony stimulating factor (CSF) for sustained grwoth. CSF consists of cell extracts from monocytes, macrophages, endothelial cells, fat cells, and other cellular elements. In the presence of CSF, colonies of granulocytes and monocytes are formed in 7–10 days. These colonies of granulocytes and monocytes have been named CFU-C (C for culture) or CFU_{GM} (GM for granulocytes and monocytes), indicating that they are committed to the production of granulocytic and monocytic cells. Other more mature and specialized precursors include CFU_{Eo} (eosinophils), CFU_{Ba} (basophils). CFU_E (erythroid), and CFU_{Meg} (megakaryocytes). BFU_E (burst-forming unit, erythroid) refers to an earlier erythroid progenitor than the CFU_E.

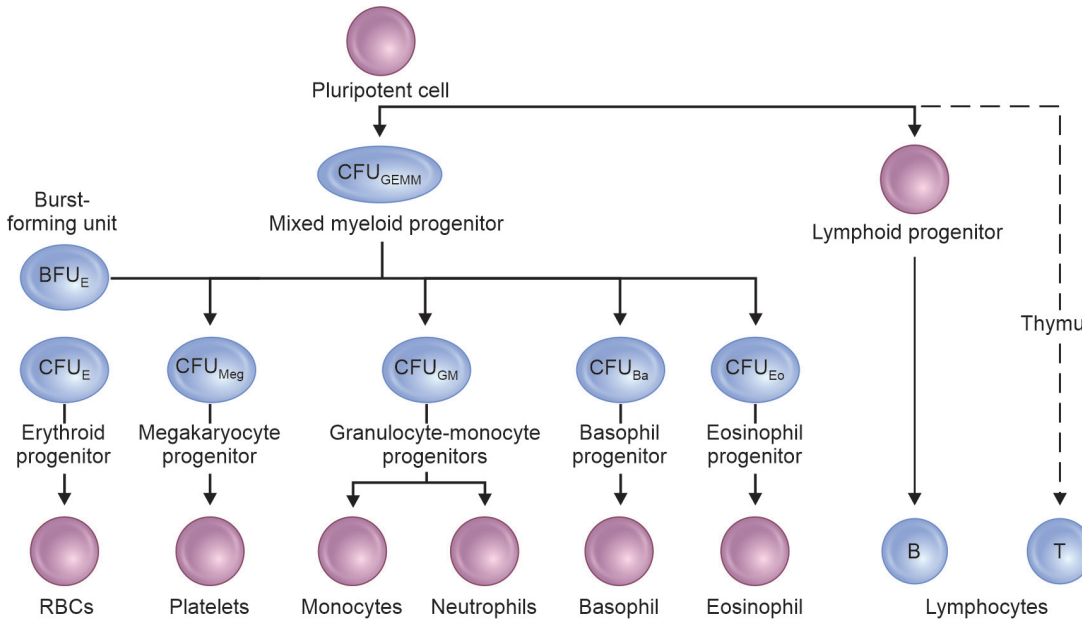


Fig. 2.2: Hematopoietic stem and progenitor cells

Stromal cells and extracellular matrix, on which hemopoiesis depends	
Stromal cells	***Extracellular matrix***
Macrophages	Fibronectin
Fibroblasts	Hemonectin
Reticular cells	Laminin Collagen
Fat cells Endothelial cells	Proteoglycans (acid mucopolysaccharides), e.g. chondroitin, heparin

Hemopoiesis occurs in a suitable microenvironment provided by a stromal matrix on which stem cells grow and divide. Proliferation of the stem and progenitor cell is under the control of the hormone-like inducers of growth and differentiation, produced by stromal cells of the microenvironment and by the hemopoietic cells themselves. For instance, the colony-stimulating factors, interleukins and erythropoietin. These may act locally or via the systemic circulation.

A large number of molecules called cell adhesion molecules (CAMs) mediate attachment of various body cells to each other and to extracellular matrix. The adhesion molecules on leucocytes and other hemopoietic cells are termed receptors and they bind with molecules (termed ligands) on the surface of target cells. The adhesion molecules are divided into three main groups according to their molecular structure: immunoglobulin superfamily, selectins and integrins.

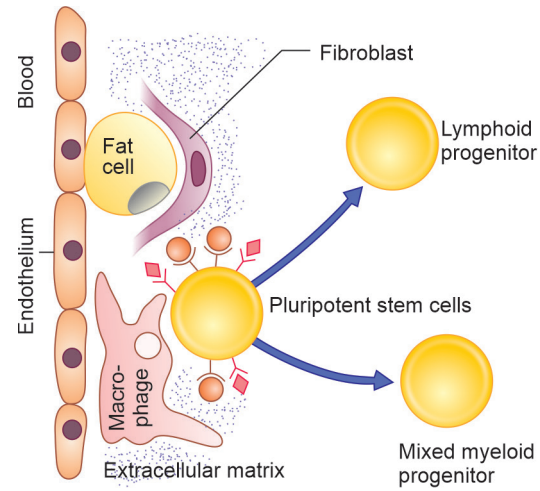


Fig. 2.3: Hemopoiesis—microenvironment

Hemopoiesis occurs in a suitable microenvironment provided by stromal matrix on which stem cells grow and divide. Adhesion molecules (—○●—) and growth factors (—≺◆—) found to stromal cells on extracellular matrix provide attachment sites for the stem cells (Fig. 2.3).

Adhesion Molecules

Family	***Function***	
Immunoglobulin superfamily	Antigen receptors, growth factor receptors	T cell receptors immunoglobulins, e.g. Kit, Fms
Selectins	Leukocyte and platelet adhesion to endothelium during inflammation or coagulation	
Integrins	a. VLA (very late activation) antigens—cell adhesion to extracellular matrix b. Leukocyte cell adhesion molecules—immune adherence c. Cytoadhesins, e.g. platelet glycoprotein 11b/111a—platelet vessel wall interaction	

Blood Formation has to Undergo Three Stages

1. Multiplication of precursor cells (1% of all marrow cells are in dividing phase)
2. Gradual maturation (both structural and functional).
3. Release into the peripheral circulation. The exact release mechanism is ill-understood, granulocytes achieve this by their motility perhaps under the effect of neutrophil releasing factor and RBCs by diapedesis, erythropoietin is said to enhance this process.

ERYTHROPOIESIS (Flowchart 2.1, Figs 2.4 and 2.5)

Mature RBCs are derived from committed erythroid progenitor cells through a series of mitotic divisions and maturation phases. Erythropoietin, a humoral agent produced mainly by the kidney, stimulates erythropoiesis by acting on stem cells to induce proliferation and differentiation of erythrocytes. Tissue hypoxia is the main stimulus for the production of erythropoietin by the kidneys.

The *in vitro* systems devised for assay of CFU-S defined two classes of erythroid stem cells. The committed myeloid stem cell CFU_{GEMM} differentiates into a primitive erythroid cell called burst-forming unit erythroid (BFU_E) which requires large amounts of erythropoietin and long culture times to form large erythroid colonies (or "bursts"). The BFU_E differentiates into an erythroid stem cell called colony-forming unit-erythroid (CFU_E) which gives rise to tiny erythroid colonies and needs low levels of erythropoietin and short culture times.

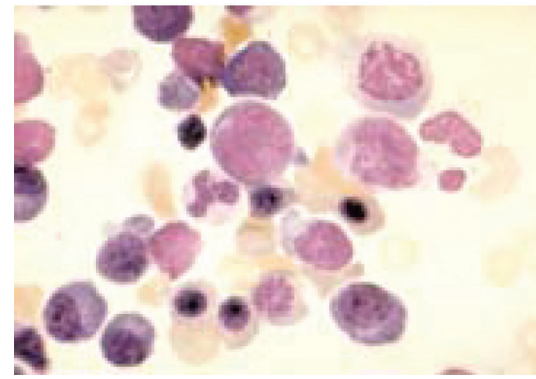
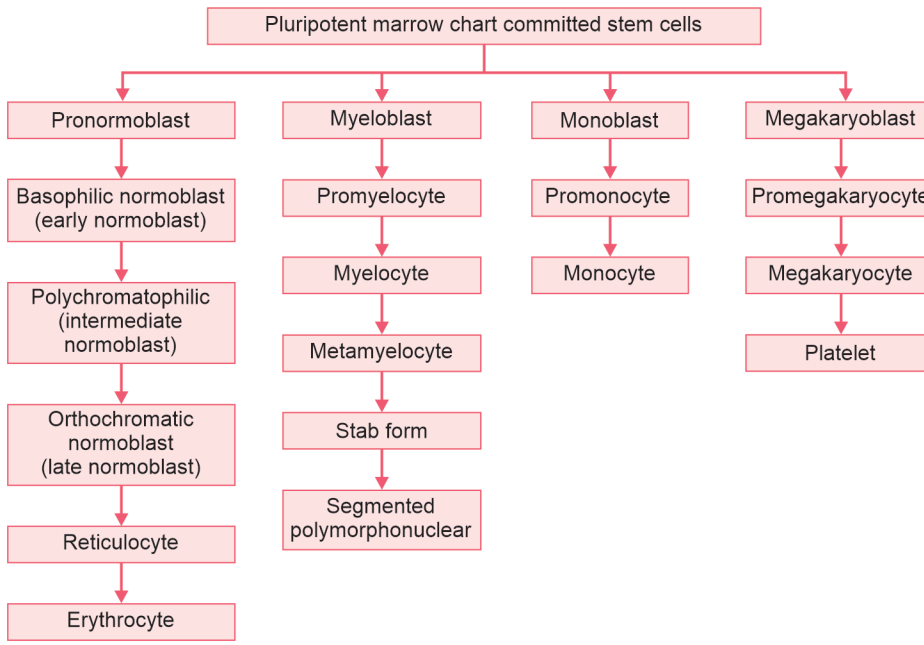


Fig. 2.4: Erythroid series (erythropoiesis)

Flowchart 2.1: Maturation chart



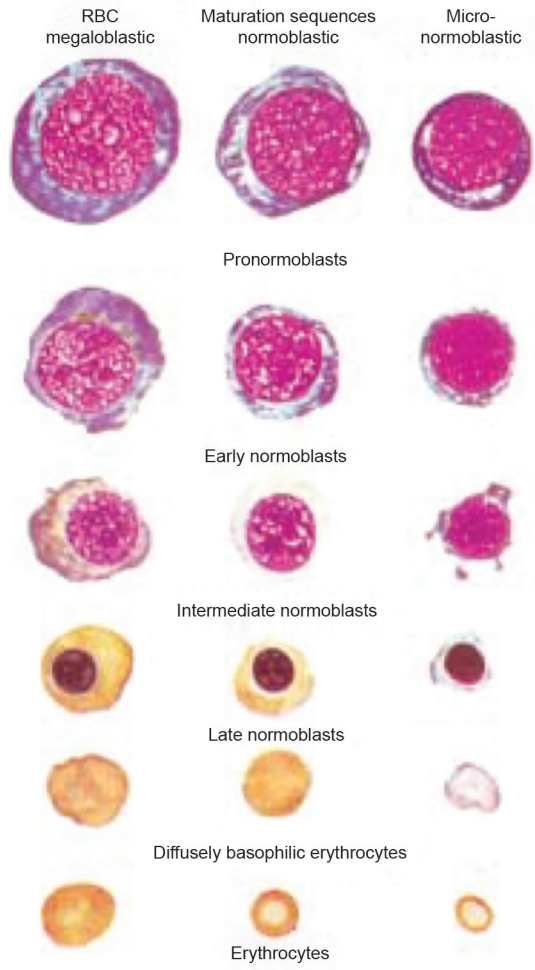


Fig. 2.5: Erythropoiesis

Erythron Concept

Blood cells markedly differ from rest of the body cells both in structure and function. With respect to erythrocytes, it is useful to conceive of the precursors and mature cells as components of a single, though discontinuous organ known as the erythron. This concept emphasizes the functional unity of the red blood corpuscles and their precursors. The insterstitial tissue of the erythron comprise the plasma and the fat and reticulum of the bone marrow.

Erythropoiesis occurs in the marrow over a period of four to five days through successive morphologic alterations in the nucleated cells from pronormoblast to the anucleate mature RBC.

- Erythroblast is a nucleated red cell.
- Normoblast implies normal (reaction) erythropoiesis.
- Megaloblast implies megaloblastic (reaction) erythropoiesis.
- Normoblastic maturation involves:
  - Reduction in cell size
  - Ripening of cytoplasm, i.e. hemoglobinization.

- Maturation time from pronormoblast to RBC is 7 days.
- Mitotic division occurs till the intermediate normoblast stage. 14–16 erythrocytes are produced from one pronormoblast. 10–15% of nucleated red cells die in marrow maturation.
- The basophilic (bluish) cytoplasm in erythrocytic precursors is due to presence of ribosomes joined together (2–8) by a strand of messenger RNA called ribosomal RNA.
- Ultrastructural (electron microscopy) differentiation from other early precursors is made by the presence of ferritin in the normoblasts.

The amount of "reticulum" decreases as the cells mature. Reticulocytes are more adhesive than adult RBCs and move about in currents at much slower rate than do mature cells. They appear to have a coating of globulin, at least part of which is transferrin. Their specific gravity is lower than that of adult RBCs, and they tend to collect in the upper portions of suspensions of corpuscles. They vary in resistance to hypotonic solutions. They have metabolic pathways that are lacking in mature red cells, including an intact tricarboxylic acid cycle.

Erythron: Proliferation and Maturation

Within the erythron, cellular maturation and proliferation proceed simultaneously. All identifiable erythroid progenitors and morphologically identifiable erythroid precursors are functionally destined to mature; thus they are incapable of self-maintenance. Maintenance of erythron is the function of the stem cell compartment. It takes about 12–15 days for a cell at BFU_E stage to mature into normoblasts, 6–8 days for BFU_E to differentiate into CFU_E, 5–7 days to reach the early normoblast stage. Probably three to five cell divisions occur along the maturation of erythroid precursors. Thus, 8–32 mature RBCs are derived from each pronormoblast cell division stops at intermediate normoblast stage, late normoblasts cannot synthesize DNA and, therefore, cannot divide.

BFU_E is totally interleukin 3 (IL-3) dependent and bears a small numbers of EPO (erythropoietin) receptors. Within 72 hours in culture, these cells become fully dependent on EPO (mature BFU_E) and in their presence proliferate and differentiate into CFU_E. At this stage EPO induces an increase in the synthesis of RNA, closely followed by the induction of β-globin gene transcription. Other biochemical events include increased uptake of calcium and glucose, synthesis of transferrin receptors, increased iron uptake hemoglobin synthesis, and appearance of erythrocyte membrane proteins. Hemoglobin synthesis continues as the cell matures further into the early normoblast stage, and at the intermediate normoblast stage enough hemoglobin has accumulated in the cytoplasm to give the cell the mild acidophilic reaction as detected by routine Romanowsky stains. Hemoglobin synthesis continues throughout the late normoblast stage and persists at a very low rate in the reticulocyte after denucleation. Mature red cells, lacking ribosomes; cannot synthesise hemoglobin.

One can observe morphologic evidence of nuclear degeneration as early as normoblast stage, by late normoblast stage nucleus becomes completely inactive, unable to synthesize either DNA or RNA. It is thought that intracellular hemoglobin brings about this cessation of nuclear activity, it gains access to the nucleus through pores in the nuclear membrane. After reaching a critical concentration, the nuclear hemoglobin may react with nucleohistones, thereby bringing about nuclear inactivation and condensation.

After the nucleus degenerates, it is extruded from the cell. The process takes about 5–60 minutes. The extruded nucleus carries with it a rim of cytoplasm, including ribosomes, hemoglobin and occasional mitochondria.

Enucleation is a process akin to cytokinesis and does not seem to depend upon either the presence of extracellular matrix proteins or accessory cells. It is also thought that the late normoblast cytoplasm and the organelles manage to squeeze through the endothelial cytoplasmic pores but the nucleus being more rigid gets caught and thus gets enucleated while rest of the cell now a reticulocyte passes through. Soon after denucleation, the nucleus is engulfed by a macrophage. This cell may remain in marrow for several days as a reticulocyte. After release the reticulocyte must be sequestered in the spleen for 1–2 days. Here additional maturation may occur, and the composition of the membrane lipids may be altered. As the reticulocyte matures to an adult erythrocyte, it loses its ability to synthesis hemoglobin. Both particulate and soluble RNA fractions are catabolised by a ribonuclease. Thus, a mature RBC is formed.

Erythroid Precursors

The least mature, recognisable erythrocyte precursor cell is known as pronormoblast or proerythroblast. The various stages of maturation in order of increasing maturity, are known as pronormoblast, early normoblast or basophilic normoblast, intermediate normoblast or orthochromatic normoblast. Ordinary Romanowsky dyes can differentiate each cell stage with light microscopy.

Cytoplasmic maturation can be assessed by the change in staining characteristics, as the deep blue color from the high RNA content of immature cells gives way to the red color characteristic of hemoglobin. Nuclear maturation is evaluated by disappearance of nucleoli and the condensation of chromatin as nuclear activity ceases.

The cytoplasm shows ribosomes which remain free in the cytoplasm as polyribosomes; these consist of eight individual ribosomes joined together by a strand of messenger RNA. Ribosomal RNA accounts for the deep blue staining that is characteristic to younger cells (pronormoblast to intermediate normoblast). Mitochondria are most numerous in earlier stages of maturation. Ferritin containing vesicles are found in the cytoplasm, these are thought to arise by pinocytosis, whereby macromolecular substances are brought into the cytoplasm. Other cytoplasmic structures include Golgi apparatus and randomly oriented occasional microtubules (Fig. 2.6).

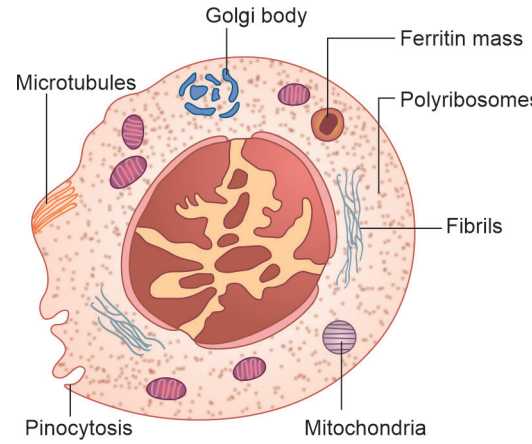


Fig. 2.6: Schematic diagram of the ultrastructure of the normoblast as visualized by electron microscopy

STAGES OF NORMOBLASTIC DIFFERENTIATION

Pronormoblast

- This is a round or oval moderate to large cell (14–20 μm)
- It has a large nucleus, occupying 80% of the cell and a basophilic cytoplasm.
- Nucleus of the youngest cell differs a little from the myeloblast. Nucleoli are present and may be prominent.
- There is a very thin or delicate membrane penetrated by pores that connect the nucleoplasm to cytoplasm.

- Electron microscopy (EM) reveals ferritin molecules, light microscopy (LM) may not reveal hemoglobin by Giemsa stain.
- As compared to myeloblasts and lymphoblasts, the cytoplasm has a tendency to be more homogeneous and condensed and may appear granular. A small pale area seen corresponds to the Golgi apparatus in the cytoplasm. The nuclear chromatin is somewhat more coarse than that of myeloblasts or lymphoblasts. A lot of mitochondria are present in the cytoplasm too.

Early Normoblast (Basophilic Normoblast or Basophilic Erythroblast)

- It is similar to pronormoblast except that nucleoli are no longer visible and the cell is little smaller (12–17 μm in diameter).
- Chromatin condensation begins at this stage. On LM, the chromatin may appear coarse and granular, and there is a little resemblance to the myeloblast.
- The nuclear structure may assume a wheel-spoke arrangement.
- Ribosomes reach their maximum number, therefore, the cytoplasm is deep basophilic, even more than the pronormoblast. The color changes in the subsequent stages reflect decreasing ribosomal RNA and increasing acidophilic hemoglobinization.

Intermediate Normoblast (Polychromatophilic Normoblast or Erythroblast)

- 12–15 in μm diameter, shows the first faint blush of hemoglobin which appears near the nucleus.
- Chromatin further condenses and irregular masses are formed, nucleoli are not visible.
- The nucleus is smaller (7–9 μm) as is the whole cell.
- As cell matures, mitochondria decrease in number.

Late Normoblast (Orthochromatic Normoblast or Erythroblast)

- 8–12 μm in diameter, cytoplasm is more or less fully hemoglobinized (color same as mature cells)
- Nucleus undergoes pyknotic degeneration, chromatin becomes greatly condensed and the nucleus shrinks.
- Nucleus may become a homogeneous mass and may assume bizarre forms. Finally the nucleus is extruded.

Reticulocyte

- When the nucleus is extruded from a late normoblast it is called a reticulocyte.
- Reticulocytes are little larger (20%) than mature RBCs. They contain certain cytoplasmic organelles, such as ribosomes, mitochondria, and the Golgi complex, and have special staining characteristics. Methanol, etc. cause a uniform precipitation of the ribosomal RNA. Such cells may appear uniformly blue or gray, or various shades of basophilia may be intermingled with pink-staining portions (polychromatophilia or polychromasia).
- Supravital stains (new methylene blue or brilliant cresyl blue) cause the ribosomal RNA to precipitate or aggregate into a network of strands or clumps that, unfortunately, have been called "reticulum". This led to them being labelled as reticulocytes. Both terms are misnomers because true endoplasmic reticulum does not exist in reticulocytes. This so-called "reticulum" decreases as the cells mature. Concentration of stain and its pH can have variable effects on the appearance of recticulocytes.
- At its latest stage it becomes the typical bilaterally indented disc shape of the mature RBC.

Control of Erythropoiesis

In broad outlines, this control system operates in the following manner. Alterations in the concentrations of hemoglobin in the blood lead to changes in tissue oxygen tension within the kidney. In response to hypoxia, it secretes a hormone called erythropoietin. This hormone induces primitive marrow cells to differentiate into pronormoblasts, thereby bringing about expansion of the erythroid marrow and an increase in red cell production. This, in turn, leads to an increase in size of the erythron and an increase in tissue oxygen levels (Fig. 2.7).

Erythropoietin

(Erythropoiesis stimulating factor, ESF) Molecular weight = 34000. It is a glycoprotein containing sialic acid, hexosamine, and hexoses. In man, kidney is the major source of erythropoietin production, but liver and some other organs also contribute.

Specialized cells producing EPO (erythropoietin) have been identified in renal and hepatic parenchyma. These rare EPO producing cells are found in the interstitium of the renal parenchyma, outside the tubular basement membrane, mostly in the inner cortex and outer medulla. They contain EPO mRNA. In liver mRNA is detected in hepatocytes. The number of interstitial renal EPO-producing cells increases in response to anemia, indicating that increased demands for EPO are met by an increase in the number of EPO-producing cells rather than by an increased synthesis of EPO by a preset number of cells. Thus, it appears that EPO is synthesized *de novo* in response to hypoxia and there is no detectable storage of the hormone. Increased levels of circulating EPO do not seem to exert a negative effect on EPO production.

Action of EPO

1. Increasing the number of stem cells committed to erythropoiesis.
   - RBC precursors have EPO receptors. They are detected on BFU_E and their density increases as the BFU_E matures to CFU_E. Erythroid cells at a stage between CFU_E and pronormoblast seem to have the highest density of EPO receptors, which decrease in

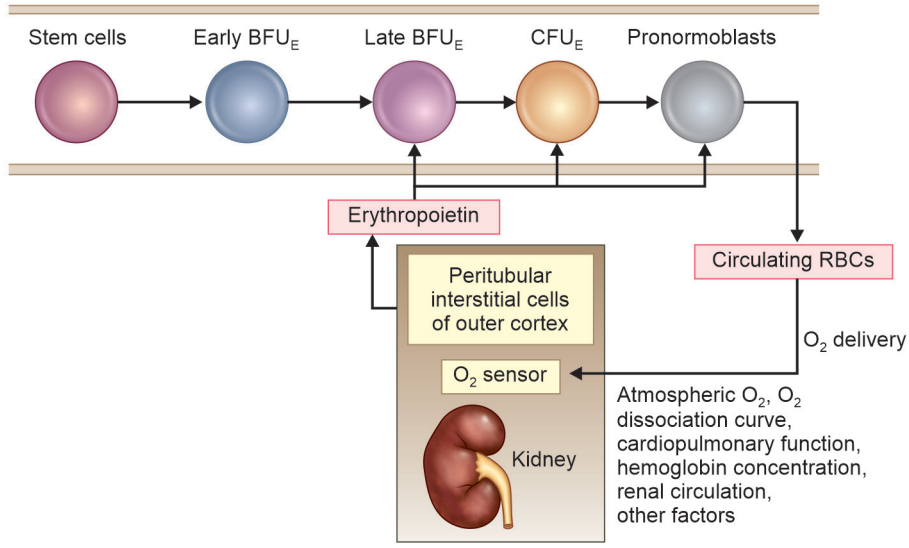


Fig. 2.7: Regulation of erythropoiesis

number as the pronormoblast matures and eventually disappear at the late normoblast stage.
- Following binding of EPO to its receptor, the hormone is rapidly endocytosed and degraded. The proportion of erythroid cells in the marrow increases and, in the chronic state, there is anatomical expansion of erythroid tissue down the long bones and sometimes in extramedullary sites. In infants, the marrow cavity may expand into cortical bone and bone deformities may result, e.g. frontal bossing and protrusion of maxilla.

2. Increasing hemoglobin synthesis in red cell precursors.
3. Decreasing maturation time of red cell precursors.
4. Releasing marrow reticulocytes into peripheral blood at an earlier stage than normal ("shift reticulocytes"), seen on peripheral smears as polychromatophilic red cells.

Substances Needed for Erythropoiesis

As the demand for new cells is great, the marrow requires large amounts of hemoglobin. The following groups of substances are needed:

1. **Metals:** Iron, manganese, cobalt.
2. **Vitamins:** Vitamin B_{12}, folate, vitamin C, vitamin E, vitamin B_6 (pyridoxine), thiamine, riboflavin, pantothenic acid.
3. **Amino acids**
4. **Hormones:** EPO, androgens, thyroxine
   Erythropoietin can be detected in both urine and plasma.

Normal Values

Normal values for urine erythropoietin in men have been reported to be 1 unit/day. Values in women are about 50% lower. With immunologic assay, normal values of plasma erythropoietin have been reported to be 7.5–60 mU per ml.

Erythropoietin levels in urine and plasma are increased in:

- Iron deficiency anemia
- Pernicious anemia
- Leukemias
- Aplastic anemia
- Sickle cell anemia and others
- Acute hypoxemia
- Hemolytic anemia
- Nephrectomy
- Kidney transplant rejection
- Renal cancer and renal cysts
- Permanent high altitude residence/pulmonary disease/hypoxia
- Polycythemia associated with certain tumours, e.g. hypernephromas, cerebellar hemangioblastomas, hepatomas, fibroid of uterus.

Erythropoietin levels in urine and plasma are reduced in:

- Anemias of renal insufficiency/chronic renal failure
- Chronic disorders (cancer, infection, rheumatoid arthritis)
- Protein calorie malnutrition
- Certain endocrine deficiency states

- Anemia associated with abnormal hemoglobins with reduced oxygen affinity, e.g. Hb Seattle
- Hodgkin's disase.

Ineffective Erythropoiesis

This term refers to the production of erythrocytes that are so defective that they are destroyed before they leave the marrow or shortly thereafter.

This occurs in:

- Myelosclerosis
- Megaloblastic anemia
- Thalassemia
- Erythroleukemia
- Sideroblastic anemia

LEUKOPOIESIS (Fig. 2.8)

MYELOPOIESIS

Neutrophilic, eosinophilic, and basophilic granulocytes are thought to follow similar patterns of proliferation, differentiation, maturation, and storage in the bone marrow and delivery to the blood. The details of these processes are best documented for neutrophils. In the first three morphologic stages, the myeloblast, promyelocyte, and myelocyte cells are capable of replication. Later stages cannot divide but continue to differentiate classifying a cell (precursor) in one category or another is difficult because it is actually a transition between the two.

Myeloid Series

The myeloid series: Specific granules are developed at the myelocyte stage which determine the nature of the mature cell (Figs 2.8 and 2.9).

Development of a Mature Neutrophil

Maturation involves:

- Development of specific granules
- Loss of basophilia of the cytoplasm
- Nuclear ripening till the segmented stage
- Ability to be motile and to phagocytose
- Mitotic division occurs till the myelocyte stage only.

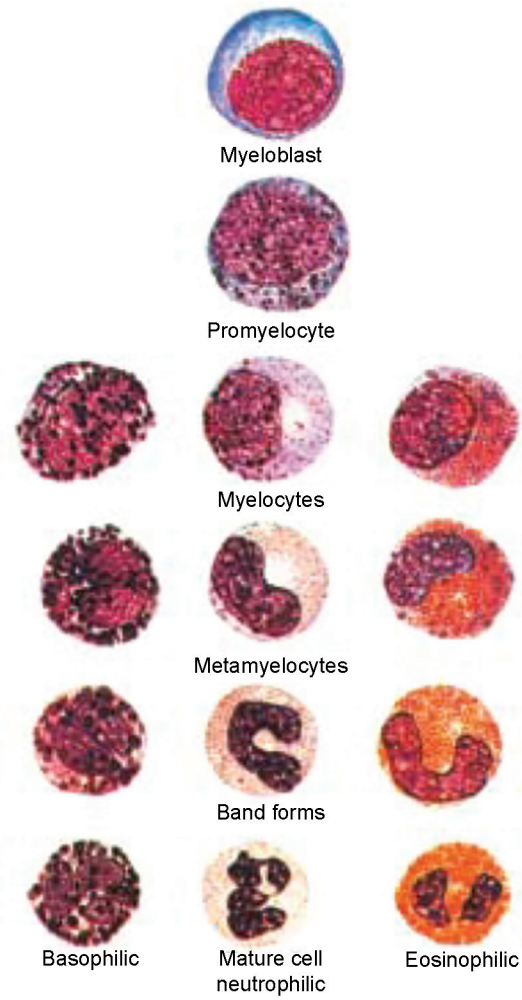


Fig. 2.8: Leukopoiesis

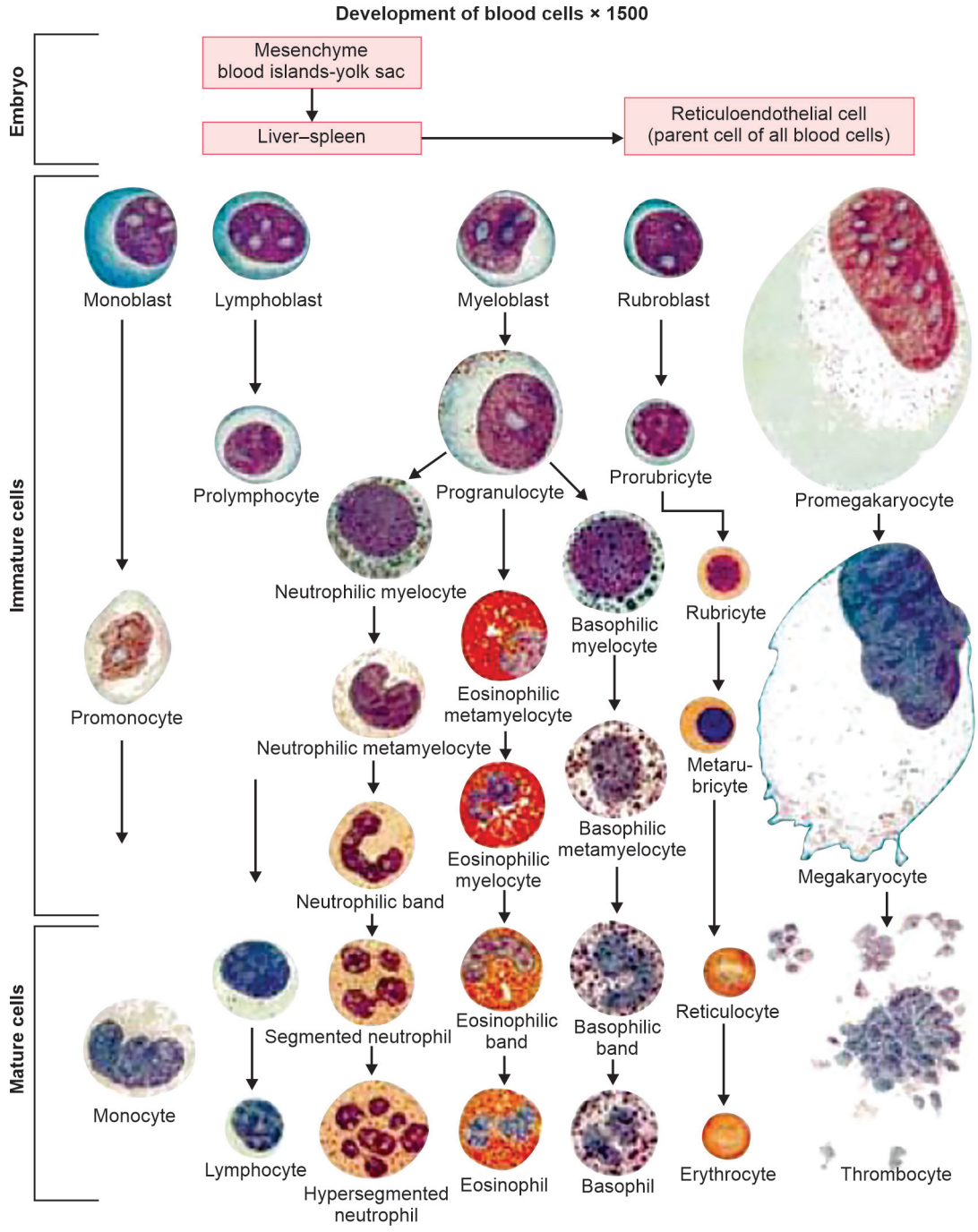


Fig. 2.9: Development of neutrophils and other cells

MYELOID SERIES

Myeloblast (Fig. 2.10A)

- An immature cell, typically found in bone marrow not in the blood. Its stem cell is CFU_{GEMM}. CFU_{GM} can divide to form promyelocytes which can give rise to myelocytes.

- Has a large nucleus, round or slightly oval and has the least amount of cytoplasm.
- The nuclear membrane is smooth and even in outline and is exceedingly thin with no condensation of chromatin near its inner surface (as seen in lymphoblasts). The chromatin shows an even, diffuse distribution with no aggregation into larger masses, although some condensation may be seen about the nucleoli. The chromatin may appear in the form of fine strands, thus giving the nucleus a fine sieve-like appearance; alternatively, it may have the form of fine dust-like granules, producing a uniform stippled effect.

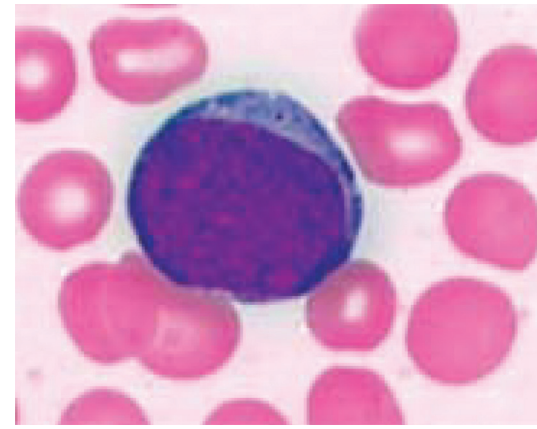


Fig. 2.10A: Myelobast

- Generally the myeloblast nucleus contains two to five pale, sky-blue nucleoli.
- The cytoplasm is basophilic (blue), and usually, although not invariably, no perinuclear halo/hoff is seen. Sometimes the cytoplasm is reticular, spongy, or foamy. By definition no granules are present in the cytoplasm.
- Leukemic myeloblasts that contain no perceptible granules often are identified by special stains that demonstrate the presence of myeloperoxidase or esterase, thus providing early evidence of differentiation.
- Electron microscopy (EM) reveals similar findings. The nuclear membrane is thin and indistinct with minimal or no chromatin condensation. The numerous particles or ribonucleoprotein in the cytoplasm produce deep blue basophilia in stained preparations. Mitochondria are abundant but small and the endoplasmic reticulum is flat and appears infrequently. The Golgi apparatus is indistinct, and no cytoplasmic granules are present. EM studies of myeloblast show peroxidase activity in the rough endoplasmic reticulum and Golgi.
- If granules are seen, the cells should be classified as promyelocytes.
- In wet films myeloblasts appear immobile, also fail to move in supravital preparations but in hanging-drop preparations, myeloblasts show a characteristic snail-like movement.
- Their size varies considerably 10–20 μm in diameter because they are in the process of growth and division.
- In patients with acute leukemia, the nucleus may show several wide and deep indentations, suggesting lobulation (Reider cells). Such myeloblasts suggest more rapid maturation on the part of the nucleus than of the cytoplasm (asynchronism of DiGuglielmo). Also in association with leukemia, Auer bodies/rods are evident in the cytoplasm of cells that otherwise look like myeloblasts. Asynchrony of nuclear and cytoplasmic differentiation (Reider cells) can be seen in monocytes, lymphocytes, myeloblasts, leukoblasts, and reticuloendothelial monocytoid cells.

Differentiation from Other Blasts

With the usual Romanowsky stains and light microscopy, distinguishing among leukoblasts (myeloblasts, lymphoblasts, monoblasts) and even pronormoblasts is extremely difficult if cells are showing beginning maturational changes (granulation or hemoglobinization) are excluded from the blast category.

In lymphoblasts, the nuclear membrane is more dense than that of the myeloblast and the chromatin is more coarse and may show some aggregation. Lymphoblasts generally have one or two nucleoli and their membrane is usually distinct. The mitochondria are short and plumper than those of myeloblasts and often assume a position close to the nucleus. The monoblast is described as showing characteristics similar to those of a mature monocyte, such as a fine chromatin, pale nucleus, and ground-glass cytoplasm with a fine, irregular border. In most of the cases, the identification of blasts is greatly aided by the company they keep (the more easily recognisable cells around them in sections or smears). With the myeloblast, the demonstration of associated promyelocytes, which show azure granulation on Romanowsky stains is presumptive evidence for the cell's identification. Special stains to detect enzymes such as peroxidase or esterase, in the blast cytoplasm before lysosomal granules appear may at times be useful in identification, especially in the classification of leukemia (Table 2.1).

Promyelocytes (Neutrophilic) (Fig. 2.10B)

- EM studies demonstrate that the azurophilic or primary granules first appear at the promyelocyte stage and can be identified on fine structural study as characteristic of the neutrophil, eosinophil, or basophil series. They do not transform into specific granules, but persist throughout the remainder of the maturation sequence and are seen in all subsequent stages, including polymorphonuclear forms.
- Neutrophilic promyelocyte is little larger on average than the myeloblast.
- It has a round or oval nucleus in which the nuclear chromatin is diffusely distributed, as in the myeloblast; in later stages, slight condensation is found around the nuclear membrane.
- Nucleoli are present, but as the cell develops, they become less prominent.

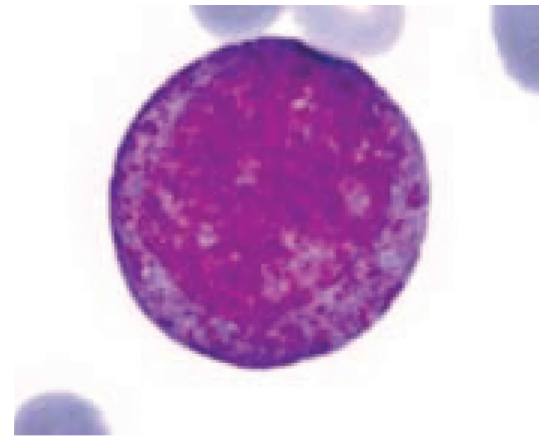


Fig. 2.10B: Promyelocyte

- Compared to the myeloblast, on EM, the endoplasmic reticulum is more prominent and takes on a dilated, vesicular appearance. The azurophilic primary granules appear and accumulate in increasing numbers during this stage, but the specific or secondary granules are not yet seen. Light microscopy may not reveal the few granules present in early promyelocytes.
- Locomotion of promyelocyte is seen slightly only at the last stage, even then, the streaming of granules so characteristic of neutrophils is lacking. The cytoplasm is supposed to be in gel form and therefore cannot change shape, consequently is unable to leave the bone marrow. In hanging drop preparations, however, promyelocytes are actively mobile.

Myelocyte (Neutrophilic) (Fig. 2.10C)

- Myelocyte is the stage when specific (secondary) granules appear in the cytoplasm and cell can be assigned to a lineage (neutrophilic) when stained and observed to have a pinkish ground-glass background color with the light microscope (LM).

- The nucleus of the neutrophilic myelocyte is usually eccentric and round or oval. One side may appear flattened.
- The nuclear chromatin is somewhat coarse, and the nucleoli are small and often not visible, although they are seen clearly on EM.
- Primary granules persist in myelocyte although their formation is limited to the promyelocyte, and each succeeding cell division leads to reduction in their number in the daughter population.

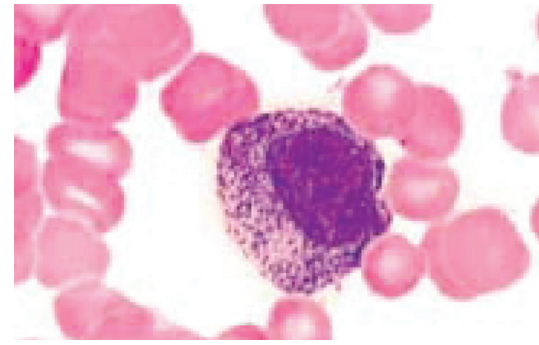


Fig. 2.10C: Myelocyte

The secondary granules are smaller than the primary granules. The amount of granular endoplasmic reticulum is lower in the myelocyte than in earlier forms, so the cytoplamsic basophilia decreases and disappears. The mitochondria remain small and are few.

Neutrophil Granule Formation

The primary granules are packed and released from the inner concave surface of the Golgi apparatus, in contrast to the specific or secondary granules of the myelocyte and later granulocyte stages that appear to be formed and released from the outer, convex surface. The ratio of secondary to primary granules in a mature neutrophil is 2–3:1.

The mature primary granules usually contain central crystalloid and apparently bind neutral red dye and are seen easily as neutral red bodies in supravital preparation. These membrane bound lysosomes contain enzymes and other substances.

Acid phosphatase activity is variable. Peroxidase has been associated with primary granules and is considered the marker enzyme for primary granules. Sulfated mucosubstance presumably accounts for the azurophilic staining of the primary granules. A significant amount of neutrophilic lysozyme is found within the primary granule.

Alkaline phosphatase is present in secondary granules in the secondary vesicle. Lysozyme is present in human neutrophil secondary granules also; about two-thirds of this antibacterial basic protein is in the secondary granule. The standard marker enzymes for specific granules are lactoferrin and B_{12}-binding protein.

A third type of granule, the tertiary granule, also known as the gelatinase granule, is synthesized mainly during the band and segmented neutrophil stages.

Metamyelocyte (Neutrophilic) (Fig. 2.10D)

- It is characterized by a clearly indented or a horseshoe-shaped nucleus without nucleoli.
- The nuclear chromatin is moderately dense, with considerable clumping evident along the nuclear membrane.
- The cytoplasm is filled with primary, secondary and tertiary granules, but the secondary granules predominate.
- The endoplasmic reticulum is sparse, as are polysomes, thus signifying the virtual completion of protein synthesis.

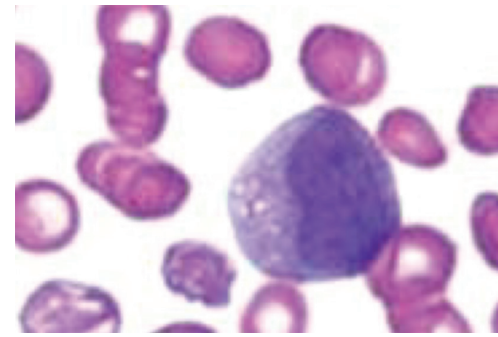


Fig. 2.10D: Metamyelocyte

Table 2.1: Morphologic classification of leukocytes (Wright–Romanowsky stain)

	Nucleus							*Cytoplasm*			
Cell type	***Size in µm***	***Position***	***Shape***	***Color***	***Chromatin***	***Nuclear membrane***	***Nucleoli***	***Relative amount***	***Color***	***Peri-nuclear halo***	***Granules***
Granulocytes											
Myeloblast	10–18	Eccentric/ central	Round/ oval	Light reddish purple	Very fine meshwork	Very fine meshwork	2–5	Scanty	Blue	None	None
Promyelocyte	12–20	Eccentric/ central	Round/ oval	Light reddish purple	Very fine meshwork	Fine	2–5	Moderate	Blue	None	Primary (azurophilic, eosinophilic or basophilic
Myelocyte	12–18	Eccentric	Oval or slightly indented	Reddish purple	Fine but becomes gradually coarser	Indistinct	Rare	Moderate	Bluish pink	None	Primary + in neutrophils, secondary or specific granules
Metamyelocyte	10–18	Central/ eccentric	Thick horseshoe or indented	Light purplish blue	Basi- and Oxychromatin clearly seen	Present	None	Plentiful	Pink	None	Neutrophilic, basophilic or eosinophilic
Juvenile/band form	10–16	Central/ eccentric	Band shape of uniform thickness	Light purplish blue	Basi- and oxychromatin clearly seen	Present	None	Plentiful	Pink	None	Neutrophilic, basophilic or eosinophilic
PMN neutrophil	10–15	Central/ eccentric	2–5 more distinct lobes	Deep purplish blue	Rather coarse	Present	None	Plentiful	Faint pink	None	Faint pink/ violet pink
PMN eosinophil	10–15	Central/ eccentric	2–3 lobes, usually 2	Purplish blue	Coarse	Present	None	Plentiful	Pink	None	Large, coarse, uniform in size, crimson red, numerous
PMN basophil	10–15	Central	2–3 lobes	Purplish blue	Coarse, overlaid with granules	Present	None	Plentiful	Faint pink	None	Large, coarse, uniform, bluish black

Contd.

Table 2.1: Morphologic classification of leukocytes (Wright–Romanowsky stain)

	Nucleus							*Cytoplasm*			
Cell type	*Size in µm*	*Position*	*Shape*	*Color*	*Chromatin*	*Nuclear membrane*	*Nucleoli*	*Relative amount*	*Color*	*Peri-nuclear halo*	*Granules*
Lymphocytes											
Lymphoblast	10–18	Eccentric/ central	Round/ oval	Light reddish purple	Moderately coarse particles, stippled	Fairly dense	1–2	Scanty	Clear blue	Present	None
Mature lymphocyte	7–18	Eccentric	Round or slightly indented	Deep purplish blue	Large masses of moderate or large size or prokaryotic	Dense	None	Scanty	Sky blue, deep blue, plentiful pink	Present if or pale is dark	None or few azurophilic cytoplasm
Monocyte/macrophage											
Promonocyte	12–20	Eccentric/ central	Round/ oval, moderately indented	Pale bluish violet	Fine reticulated, skein-like or lacy	Present	1–2	Moderate	Grayish or cloudy blue	None	Few, fine, lilac or reddish
Monocyte	12–20	Eccentric/ central	Round, oval, notched or horseshoe	Pale bluish violet	Fine reticulated, skein-like or lacy	Present	None	Abundant	Grayish or cloudy blue	None	Abundant, fine, lilac or reddish blue
Macrophage	15–80	Central indented	Elongated	Pale bluish or oval	Spongy	Distinct	None	Usually abundant	Opaque sky blue	None	Numerous, moderately coarse azure granules, and vacuoles

- Metamyelocytes do not synthesise DNA (myelocytes do).
- In differentiating metamyelocytes from myelocytes, nuclear shape is less important, the observer should pay particular attention to evidence in nucleus and cytoplasm that protein synthesis has decreased or stopped. This determination is made on the basis of the fact that the nuclear chromatin is coarse and clumped and that the cytoplasm is faint pink and is essentially the color of the mature cell in stained preparations. These features are also helpful in differentiating metamyelocytes from monocytes because in monocytes, nuclear chromatin remains fine and evidence of protein synthesis persists. Directional amoeboid movement can be shown even in coverglass slide preparations, at the metamyelocyte stage.

Band Neutrophils (Fig. 2.10E)

- This is characterized by further condensation of nuclear chromatin and transformation of nuclear shapes into sausage or band forms that have more or less uniform diameters throughout their length. Subsequently one or more constrictions begin to develop and progress until the nucleus is divided into two or more lobes, connected by filamentous strands of heterochromatin, the polymorphonuclear stage.
- A clear and easily recognisable separation between nuclear lobes is needed to count nuclear lobes for diagnostic purposes, as in the early detection of folic acid deficiency or in assessing marrow release of young forms into the blood. For such purposes, the clear separation of nuclear lobes is taken as the criterion for inclusion in the polymorphonuclear category. Cells without this complete formation of distinct lobes (usually connected by a filamentous strand) are classified as band forms.

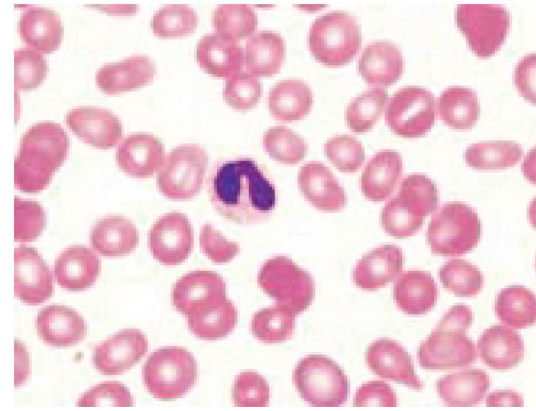


Fig. 2.10E: Band neutrophil

Polymorphonuclear Neutrophils (Fig. 2.10F)

- The nucleus in Romanowsky stained preparations is a deep, purplish color and contains course, condensed chromatin.
- The lobes are joined by thin filaments of chromatin, although filaments may not be visible if lobes are partially superimposed.
- The cytoplasm is faint pink and contains fine, specific granules that sometimes give a ground-glass appearance.
- The azurophilic or primary granules have usually lost their dark-staining characteristics by this stage, but can be seen with EM. With EM, the granules exhibit considerable variation in density, presumably a reflection of variation in enzyme content. Large masses

Fig. 2.10F: Neutrophil

of glycogen become evident for the first time in mature neutrophils; this finding may contribute to their capacity for anaerobic metabolism.

- Why nuclear lobulation? Perhaps because of loss of nucleolar emptying and has no function or perhaps it enhances cell deformability and movement through vessel walls and into sites of inflammation.
- Nuclear lobulation continues as the cell ages.
- In wet films marked ameboid activity of polymorphonuclear neutrophils at physiologic temperatures is characteristic. In supravitally stained films, the (specific) granules appear yellowish, pink and are refractile; in addition occasional larger, nonrefractile, deep red vacuoles may be seen.

Senile polymorphonuclear leukocytes are no longer motile and fail to take up the neutral red stain. They are seen in small numbers in the blood, where their survival time is short.

EOSINOPHILS (Fig. 2.10G)

- Eosinophils exhibit the same type of maturation process as neutrophils, with the exception that only one type of granule is recognized. These homogeneous granules appear to be formed throughout all the subsequent stages of maturation. Their contents are first seen as flocculent material in Golgi saccules, then in small vacuoles that condense to form the large homogeneous, dense granules. Later, crystalloids develop and the granules acquire an angular shape; the angular configuration predominates in the mature eosinophils, and the lobes are larger than those seen in neutrophils. It is unusual to find more than two lobes in mature eosinophils. Presence of more than two lobes suggests cell activation, as occurs in parasitic diseases. Eosinophilic granules are considerably larger than neutrophilic granules, appear somewhat refractile under light microscope, and stain a brilliant yellowish red with Romanowsky stains.
- Human eosinophilic granules contain eosinophil peroxidase, a heme protein that is distinct from neutrophil myeloperoxidase; they contain several cationic proteins, including eosinophilic cationic protein (ECP), and eosinophil major basic protein (MBP). MBP constitutes a major proportion of total granule protein and damages several parasites such as schistosomes, *Trichinella spiralis* larvae, and trypanosomes as well as many type of mammalian cells, including respiratory epithelium. The granules also contain eosinophil derived neurotoxin (EDN). Of the mature granulocytes, eosinophils display the most intense staining with peroxidase.

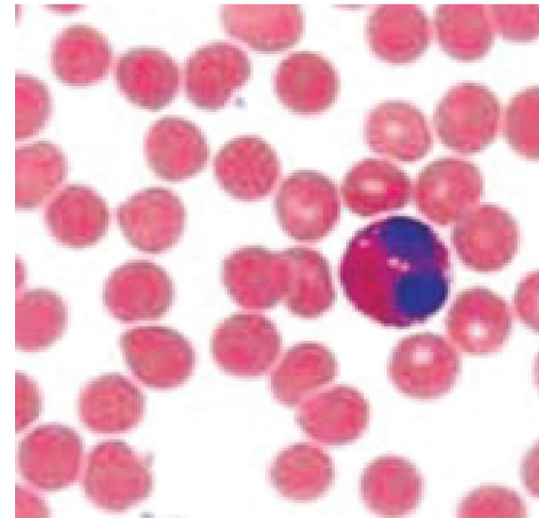


Fig. 2.10G: Eosinophil

BASOPHILS AND MAST CELLS (Fig. 2.10H and I)

- Basophils play an important role in allergic reactions. They express a unique surface antigen profile and contain a variety of vasoactive and immunomodulatory chemicals that are released on activation.

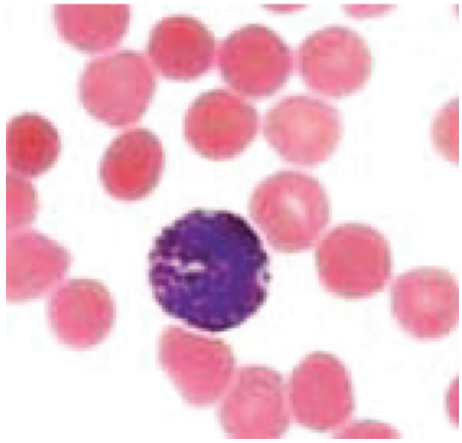


Fig. 2.10H: Basophil

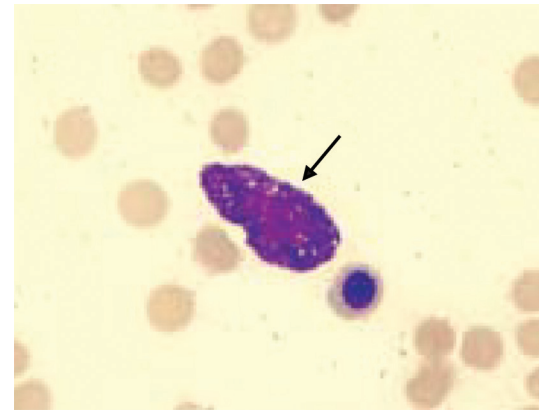


Fig. 2.10I: Mast cell (larger one)

- Basophils are distinguished by their large, coarse, purplish black granules that usually fill the cytoplasm and often obscure the nucleus.
- The granules are water-soluble and thus may be dissolved in the process of staining and washing; the cells may appear vacuolated with only a few or no basophilic granules remaining.
- The primary basophil granules are peroxidase positive and contain large amounts of heparin and histamine (sulfated acid mucosubstance), features that are probably responsible for the affinity of granules for basic dyes. These granules also contain the slow-reactive substance of anaphylaxis, kallikrein, an eosinophil chemotactic factor and a platelet activating factor. Charcot-Leyden crystals are also seen with both phase and EM, major basic protein (MBP) is found both in eosinophils and basophils. A second smaller, peroxidase negative-granule has also been identified.

Mast cells: Similar to basophils but somewhat larger cells found in the tissues are mast cells. They do not circulate in blood however, and probably mature locally in the tissues from mitotically active precursors. Although these cells resemble basophils in their metachromasia, acid nature, and content of heparin and histamine, they contain hydrolytic enzymes, 5-hydroxytryptamine (5-HT) and serotonin, which basophils do not.

LYMPHOCYTIC SERIES

Lymphoblast

10–18 μm, resembles myeloblast, cytoplasm is agranular and moderately basophilic. Nuclear chromatin gives fine reticular appearance with up to 2 nucleoli. It is peroxidase negative (Fig. 2.11A).

Prolymphocyte

Prolymphocyte has an intermediate chromatin pattern that has begun to clump in some areas of the nucleus. Nucleoli are less distinct. The differences are very subtle and in case of doubt should be called a lymphocyte (Fig. 2.11B).

Large Lymphocyte

16–22 μm, abundant pale sky blue cytoplasm with a few purplish red granules seen in about 33% of the cells (Fig. 2.11C).

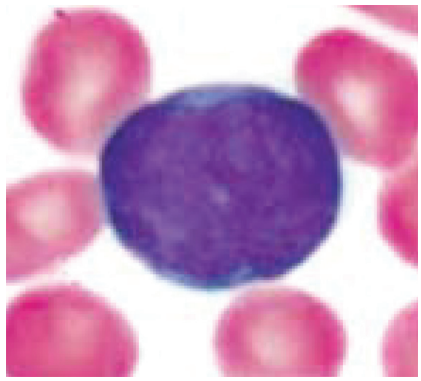


Fig. 2.11A: Lymphoblast

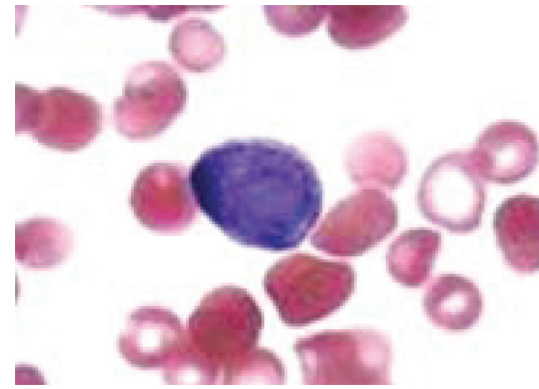


Fig. 2.11B: Prolymphocyte

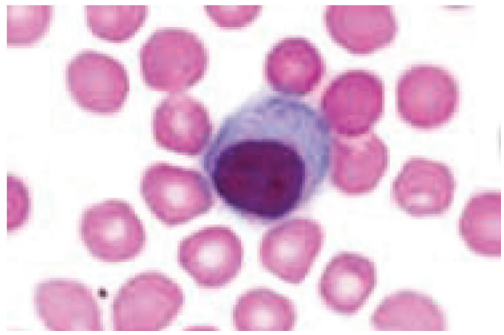


Fig. 2.11C: Large lymphocyte

Fig. 2.11D: Small lymphocyte

Small Lymphocyte

7–12 μm, scanty cytoplasm. Nucleus usually round and shows heavily clumped chromatin (Fig. 2.11D).

Further Embryological Subdivision

- T cells
- B cells
- Non-T non-B cells or NK cells
- D cells

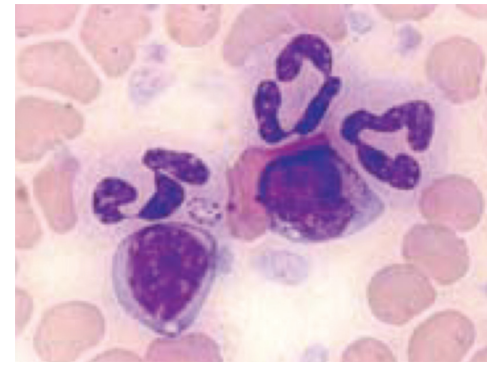


Fig. 2.11E: Reactive lymphocytes showing 3 neutrophils and 2 reactive lymphocytes

Activated and Reactive Lymphocytes

On stimulation by appropriate antigen, lymphocytes transform into immunologically competent cells. They are usually large. The increase in size is due to an increase in DNA in nucleus and of RNA in the cytoplasm, nuclei may be indented with an intermediate chromatin pattern. Some lymphocytes may show blast-like nuclei with nucleoli. Varying degrees of basophilia may be seen. There may be distinctive reddish granules. The cytoplasm may be bubbly and may show vacuoles. The most strking such lymphocytes are seen in infectious mononucleosis but reactive (or stress) lymphocytes may be seen in virus-related diseases and conditions such as post-transfusion reactions, organ transplants, and serum sickness (Fig. 2.11E).

Plasmocytes

Plasmocytes and lymphocytes are closely related. Progenitor of both, live in lymphoid organs. Both the types of cells have morphologic features and immunologic functions that are distinct from those of nonlymphoid cells. They are essential in body defence against bacteria, viruses, and other microorganisms.

Plasma cells are not seen in peripheral blood but constitute about 1% of nucleated bone marrow cells. In viral infections they may be found in peripheral blood too. Size ranges from 15 to 25 µm. May be round or oval with slightly irregular margins. The cytoplasm is nongranular and usually stains a deep or vibrant blue which has been described as "cornflower" or "larkspur" blue. They show a perinuclear halo/hoff where the Golgi apparatus is housed, fibrillar structures and vacuoles may also be seen in the cytoplasm. The nucleus is relatively small, round or oval and eccentrically placed. Nuclear chromatin is clumped in a way that it gives a "clockface" pattern. After stimulation by antigen some B cell lineage lymphocytes may be transformed into plasmacytoid cells and secrete and manufacture immunoglobulins. The immunoglobulins produced by plasma cells produce unusual morphologic variants. The proteinaceous material is in the form of round globules that my be red ("Russell bodies"), pink, blue or colorless. The globules may fill the cytoplasm giving it the appearance of bunch of grapes.

In some cells the red color has a diffuse distribution producing so-called flame cells. This red color is due to abundant IgA within the plasma cells.

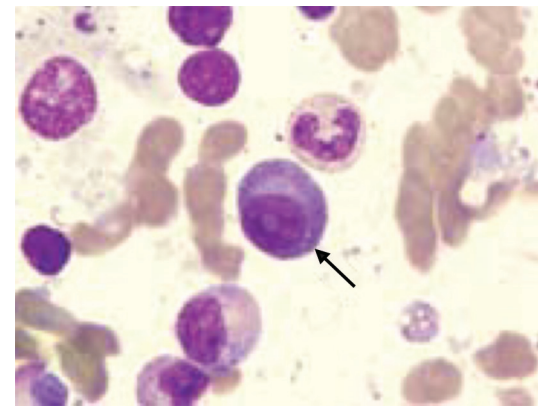


Fig. 2.11F: Plasmablast (arrow marked)

Plasmablasts and Proplasmocytes (Fig. 2.11F to H)

The plasmablasts are similar to other blasts of other series. The nuclei are large in relation to cytoplasm, appear round with linear chromatin strands and have a nucleolus. The cytoplasm is dark blue and nongranular. The blasts are identified by the presence of proplasmocytes and plasma cells around them.

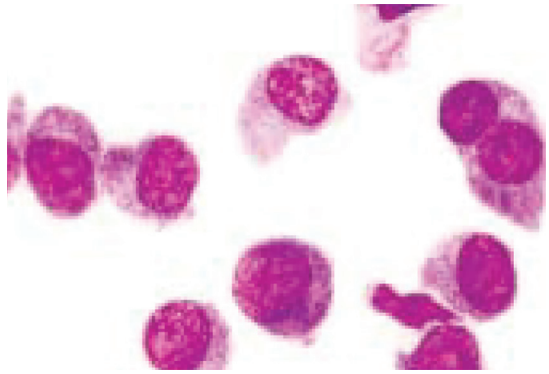


Fig. 2.11G: Proplasmocyte

Fig. 2.11H: Plasma cell

The major differences between a plasmablast and proplasmocyte is the perinuclear halo/hoff, an intermediate chromatin structure, an eccentric nucleus, and an ill-defined or absent nucleolus in proplasmocytes.

MONOCYTIC SERIES (Figs 2.11I to K and 2.12)

Monoblast

It is like a myeloblast (Fig. 2.11I).

Promonocyte

Up to 20 µm, large convoluted nucleus, chromatin in skein-like strands. Cytoplasm is dull grey-blue, may contain a few azurophilic granules (Fig. 2.11J).

Monocyte

15–20 µm, abundant dull grey-blue cytoplasm with a ground glass appearance and may show vacuolation and fine azurophilic granules. It has a kidney-shaped nucleus (Fig. 2.11K).

Fig. 2.11I: Monoblast

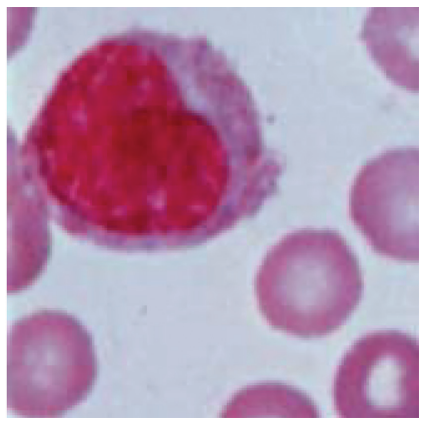


Fig. 2.11J: Promonocyte

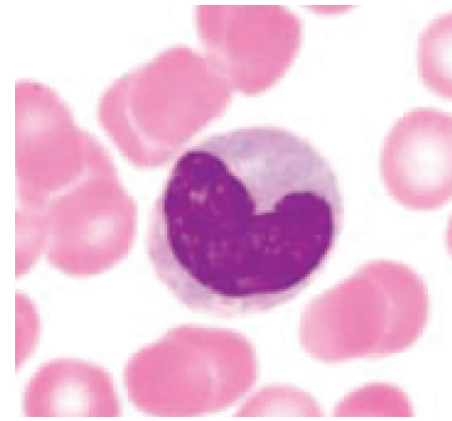


Fig. 2.11K: Monocyte

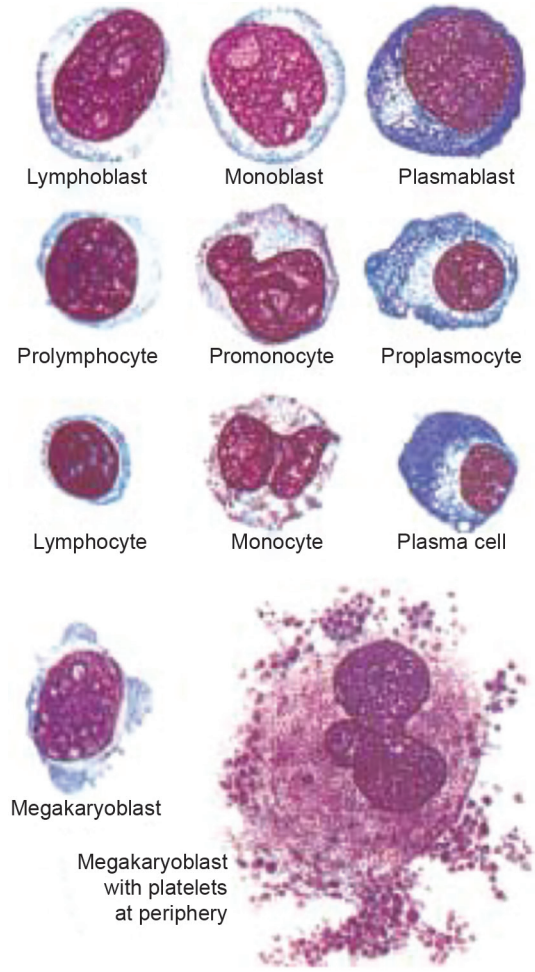


Fig. 2.12: Lymphocytic, monocytic and plasma cell series

MACROPHAGES

As monocytes grow, they become too large to pass readily through capillaries. Monocytes then migrate into tissues and mature into activated macrophages in many organs (e.g. alveolar macrophages, splenic macrophages, Kupffer's cells). Macrophages do not normally re-enter the bloodstream.

The monocyte-macrophage system plays a key role in host defense. Phagocytes entrap and kill pathogenic microorganisms, ingest and degrade noxious external agents, engulf and process antigens, and pass on processed antigenic substances to cells of the lymphocytic plasma cell system responsible for synthesis of antibodies and for cell-mediated immunity. Phagocytic cells remove from circulating blood hemoglobin-containing red cells, injured and dead cells, hemosiderin granules, white and red cell fragments, insoluble particles, activated clotting factors, and antigen–antibody complexes. Motile macrophages, escaping between epithelial cells of respiratory tracts and gastrointestinal and genitourinary organs, perform a scavenger function by clearing the body of unneeded debris. When activated, some macrophages are able to kill some types of tumor cells in culture. Macrophages also serve as secretory cells by releasing IL-1, a soluble factor that induces T cell proliferation, and by elaborating prostaglandins, which are mediators of inflammation.

There is no large reserve pool of monocytes in marrow. They constitute about 2% of nucleated marrow cells. Monocytes leave the marrow when mature and enter the bloodstream where they circulate for about 14 hours before entering tissues.

Control of Leukopoiesis

Not very well understood perhaps by a feedback mechanism.

LYMPHOKINES AND MONOKINES

These are glycoproteins, which are released by lymphocytes and monocytes (macrophages), have wide ranging effects on hemopoiesis, the immune response, response to infection and to invasion by tumors. They have a complex network of interactions and are briefly desribed below (Table 2.2).

Interleukin-1 (IL-1)

IL-1 is produced by activated macrophages, and also by endothelial cells, astrocytes, fibroblasts and T cells, in two forms, alpha and beta, in the ratio of 1:10. Both α and β IL-1 are biologically active, with widespread activities in the recruitment of cells involved in the inflammatory response, in wound healing, in the immune response and in stages of hemopoiesis. IL-1 acts as an endogenous pyrogen; activates lymphocytes, neutrophils, other macrophages and natural killer cells; induces proliferation of osteoclasts, fibroblasts, epithelial, endothelial and synovial cells and enhances MHC class II antigen expression. Prostaglandin and collagenase synthesis is increased. An important role in hemopoiesis is stimulation of marrow stromal cells to secrete CSFs. CSFs ultimately induce hemopoiesis.

Interleukin-2 (IL-2)

This promotes the proliferation of T lymphocytes and, to a lesser extent, B cells and monocytes, it promotes cytotoxic function by stimulating the proliferation and activity of natural killer (NK) cells. The IL-2 receptor consists of two proteins.

Interleukin-4 (IL-4)

IL-4 has wide variety of effects in hemopoiesis and in the development of both B and T cells. It is needed for developing B cells to switch expression of immunoglobulin class.

Interleukin-6

This has a wide variety of effects on hemopoiesis, the immune system and as an acute phase protein. It is also necessary for platelet production.

Interleukins 7 and 9–13

These are more recently discovered cytokines, have roles in hemopoiesis and immune system development and function.

Interferons

These are produced in response to viral infections. They inhibit viral replication in other cells by inducing new cellular RNA and protein synthesis. Interleukins activate lymphocytes to produce interferon.

Lymphocytes

Lymphocytes assist the phagocytes in the defence of the body against infection and other foreign invasion and add specificity to the attack. They are produced in the bone marrow

from pluripotent stem cells and fall into two main groups with different functions, T cells and B cells indistinguishable by routine Romanowsky stains. T cells are processed initially in the thymus; B cells are so-called because in birds they differentiate in an organ known as bursa of Fabricius.

T Cells

Mature T cells, which constitute 65–70% of the circulating lymphocyte population, carry a marker (antigen CD2) which binds sheep erythrocytes. Other surface markers may be defined by indirect immunofluorescence or by immunoperoxidase-linked specific antibodies, some of which define subpopulations of T cells, T cells are subdivided into two major subsets which are detected by monoclonal antibodies to CD8 and CD4 surface membrane antigens. CD8+ cells, the major subpopulation of T cells in the marrow, include suppressor/cytotoxic cells, while CD4+ (helper) cells predominate in the peripheral blood.

T cells also contain a number of lysosomal acid hydrolases, such as β-glucosidase, and acid phosphatase, which may be detected cytologically as discrete masses in the Golgi zone in the cytoplasm.

B Cells

They comprise 5–15% of the circulating lymphocyte population. Mature B cells are defined by the presence of endogenously produced immunoglobulin molecules inserted into the surface membrane where they act as receptors for specific antigens. The immunoglobulins (Ig) may be one of the five classes; IgM, IgD, IgG (divided into four subtypes), IgE or IgA (divided into two subtypes). Each immunoglobulin molecule consists of light chains (k or λ) and heavy chains (μ, δ, γ, ε, α, respectively) which determine the class of immunoglobulin. Both heavy and light chains contain constant and variable regions.

Natural Killer (NK) Cells

There is a minor population of lymphocyte cells which do not carry markers of either T or B cells and which are known as 'non-T, non-B'cells or third population cells. The majority of 'null cells' appear as large granular lymphocytes in the peripheral blood. This population of cells contains the majority of natural killer (NK) cells, which can kill target cells. They can kill tumor cells and virus infected cells. They are also involved in graft rejection. Small number of circulating 'null' cells are immature T or B cells and myeloid or erythroid progenitor cells. NK cells probably include cells of mainly T cells (CD8+) and myeloid lineage cells. Their proliferation is stimulated by interleukin-2 and interferon.

Lymphocyte Proliferation and Differentiation (Fig. 2.13)

T and B cells proliferate and develop in reactive lymphoid tissue, for instance, lymph nodes, and lymphoid tissues of alimentary and respiratory tract and spleen. Both T and B cells acquire receptors for antigens, which commit them to a single antigenic specificity and are activated when they bind their specific antigen in the presence of accessory cells.

The stimulated T and B cells proliferate and differentiate under the stimulus of factors released from antigen-presenting cells (IL-1, IL-6 and IL-7) and activated T-helper cells (IL-2, IL-4 and IL-9). The T-helper lymphocytes also secrete cytokines, especially IL-2 and IL-4 which cause B cells to multiply, to produce antibodies and to stop replication. Clones of both effector and memory B cells are produced. When the memory cells are stimulated at a later date by their specific antigen, they are able to proliferate again in an accelerated fashion (secondary response).

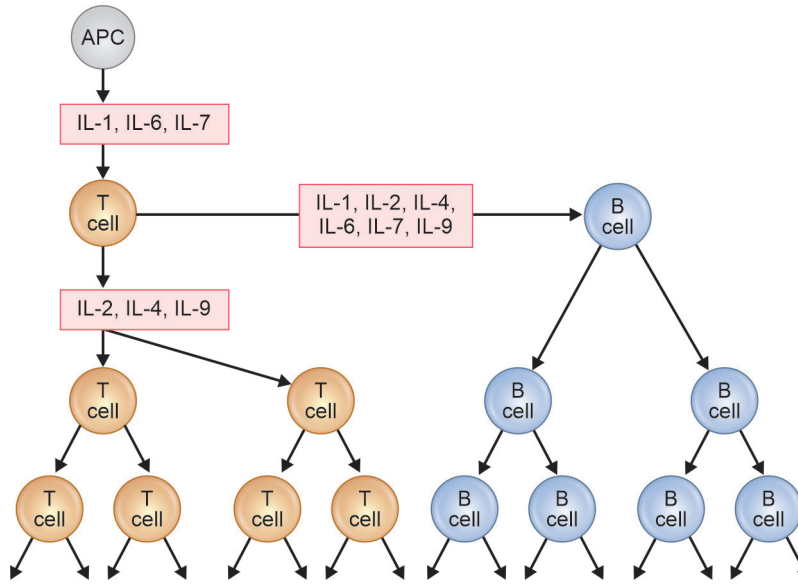


Fig. 2.13: Lymphocyte proliferation and differentiation

Activated T cells become responsible for cell-mediated immunity and secrete a number of lymphokines including interleukins 2, 3 and 5, interferons α and γ, migration inhibition factor, lymphotoxins and tumour necrosis factor. Other lymphokines activate killer T cells, enabling them to attack an invading organism or cell, induce macrophages to stay at the site of infection and help to digest the cells they have phagocytosed. They may also have a direct action on organisms by inhibiting proliferation or activating apoptosis (programmed cell destruction).

T helper cells are important in initiation of a B cell response to antigens: T suppressor cells reduce the B lymphocytic response, and T cytotoxic cells are capable of directly damaging cells recognized as foreign or virus infected.

Activated B cells are responsible for humoral immunity. Many B cell blasts mature into plasma cells which produce and secrete antibodies of one specificity and immunoglobulin class.

Lymphocyte Circulation

Lymphocytes from the primary lymphoid organs of the marrow and thymus migrate via the blood through postcapillary venules into the substance of lymph nodes, into unencapsulated lymphoid collections of the body and into the spleen. T cells home to the paracortical areas of these nodes and to the periarteriolar sheaths of the spleen. B cells accumulate selectively in germinal follicles of lymphoid tissue, in the subcapsular periphery of the cortex and in the medullary cords of the lymph nodes. Lymphocytes return to the peripheral blood by the efferent lymphatic system and the thoracic duct. The median duration of complete circulation is about 10 hours. The majority of circulating cells are T cells. B cells are mainly sessile and spend long periods in lymphoid tissue and spleen. Many lymphocytes have long lifespan and may survive as memory cells for several years.

PLATELET SERIES

Megakaryoblast

20–30 µm, has a large, oval or kidney-shaped nucleus with several nucleoli. Possesses relatively small amount of agranular cytoplasm (Fig. 2.14A).

Promegakaryocyte

Diameter 30 µm, cytoplasm intensely basophilic with fine azurophilic granules. Nucleus may show mild lobulation, chromatin is denser (Fig. 2.14B).

Megakaryocyte

30–90 µm, contains a single multilobulated or indented nucleus. Nuclear lobes vary from 14 to 16. Cytoplasm is bulky, light blue with fine azurophilic granulation. Margin is irregular and may show fragmentation or budding, a precursor of circulating platelets (Fig. 2.14C).

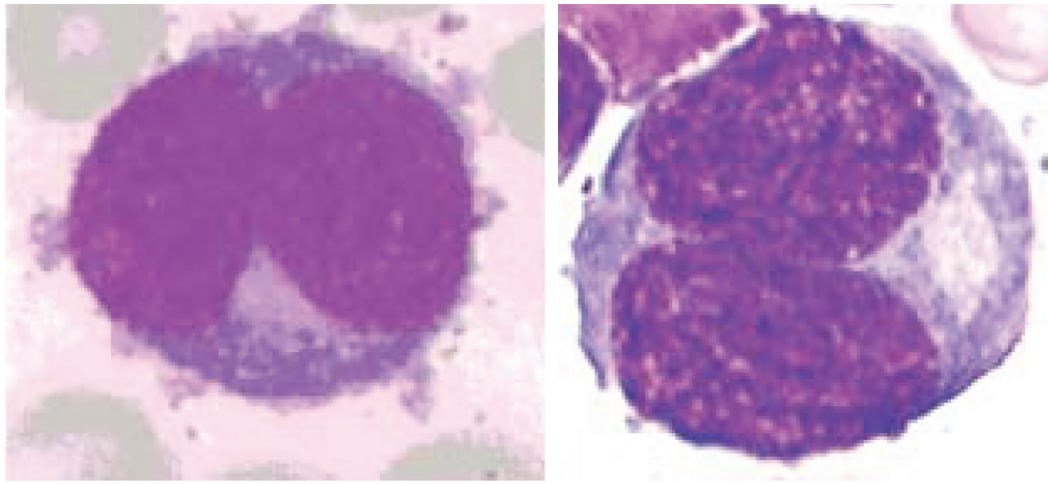


Fig. 2.14A: Megakaryoblasts

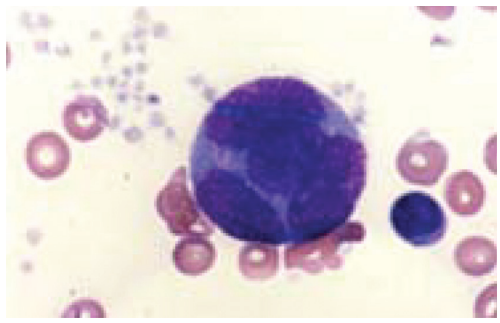


Fig. 2.14B: Promegakaryocyte

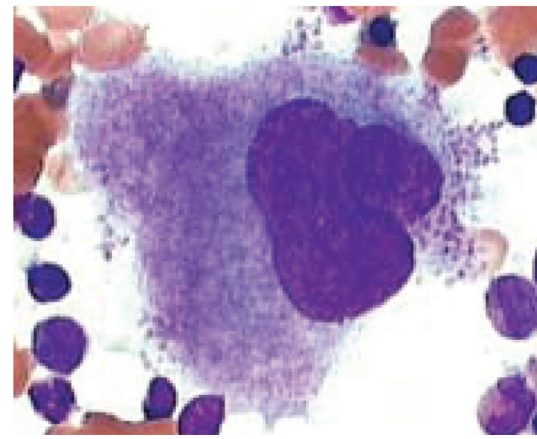


Fig. 2.14C: Megakaryocyte

Table 2.2: Biological aspects of cytokines and growth factors

Cytokine	*Molecular weight*	*Source cells*	*Target cells*	*Chief effects*
IL-1α, β	17,500	Macrophages, somatic cells	Hemopoiesis immune system	Inflammatory
IL-2	15,500	T lymphocytes	T and B cells	T cell growth factor
IL-3	28,000	T lymphocytes	Early hemopoietic cells	Hemopoietic growth factor
IL-4	19,000	T cells, mast cells	T and B cells, early hemopoietic cells	Immune and hemopoietic growth factor
IL-5	40000–50000	T cells, mast cells	Eosinophils	Eosinophil growth and differentiation factor
IL-6	21000–28000	Fibroblasts, T cells, macrophages, somatic cells	B cells, megakaryocytes	Immunoglobulin production, hepatic acute phase protein platelet production
IL-7	25000	Stromal cells	Early B and T lymphocytes	Growth factor for B and T cells
IL-8	6000–8000	Ubiquitous, macrophages, endothelial, fibroblasts	Chemoattractant for neutrophils and T lymphocytes	Neutrophil migration
IL-9	30,000–40,000	T cells	Mast cells, erythroid progenitors	Hemopoiesis and T cells development
IL-10	18,000	T helper and B cells, keratinocytes, macrophages	B cells and T cells, monocytes, macrophages	Mast cell and B cell proliferation and antibody production; suppresses cytokine production for monocytes, T helper cells
IL-11	23,000	Fibroblasts, stromal cells	Megakaryocytes, early hemopoietic cells, B cells	Hemopoiesis, thrombopoiesis, B cell immunoglobulin secretion
IL-12	70,000	Macrophages, B cells	T cells, NK cells	Induces γ interferon (IFN) and CMI
IL-13	10,000	T cells	Monocytes, B cells	Promotes IgE production
IFN-α, β	18,000–20,000	Leukocytes, fibroblasts	Macrophages, NK cells	Antiviral, antiproliferative and immunomodulating; induces class I MHC antigens
IFN-γ	20,000–25,000	T lymphocytes, NK cells	T and B lymphocytes	Immunomodulating anti-proliferative (inhibits apoptosis) and antiviral. Induces cell membrane antigens, e.g. MHC class I or II
TNF-α	17,000	Macrophages, somatic cells, T and B lymphocytes	Ubiquitous	Inflammatory, immunoenhancing and tumouricidal secondary production of cytokines by stromal cells
TNF-β	18,000	T lymphocytes (lymphotoxin)	Ubiquitous	Inflammatory, immunoenhancing, and tumouricidal secondary production of cytokines by stomal cells

Contd.

Table 2.2: Biological aspects of cytokines and growth factors

Cytokine	*Molecular weight*	*Source cells*	*Target cells*	*Chief effects*
TGF-β	25,000	Platelets, T cells, somatic cells	Fibroblasts, many other cells	Anti-inflammatory, wound healing, bone remodelling
MIP-1α			Stem cells	Inhibitory factor
SCF (kit ligand)	20,000–35,000	Stromal cells, fibroblasts, hepatocytes	Mast cell early pluripotential cells and myeloid, lymphoid or erythroid precursors	Growth factor for stem cells, early lymphoid and myeloid progenitors, mast cells
GM-CSF	14,000–35,000	Stromal cells, T lymphocytes, mast cells	Hemopoietic progenitors and late cells in granulocytic, monocytic lineages	Stimulates early hemopoiesis, mast granulopoiesis, monocyte formation and function
G-CSF	18,000	Stromal cells, macrophages, monocytes	Granulocyte progenitors and granulocytes	Early hemopoiesis, granulocyte production and function
M-CSF	40,000–70,000	Stromal cells, macrophages, monocytes	Monocytes, progenitors of monocytes	Monocyte production and function
Erythro-poietin (EPO)	30,400	Kidney, liver	Erythroid progenitors (late BFU_E, CFU_E) and erythrocyte precursors to reticulocyte stage	Growth factor for red cells

Mature Platelet

1–4 μm, formed by fragmentation of megakaryocytic pseudopods. In circulation they acquire a discoid shape. Cytoplasm stains light blue and contains purple reddish granules which may be clumped centrally (Fig. 2.14D).

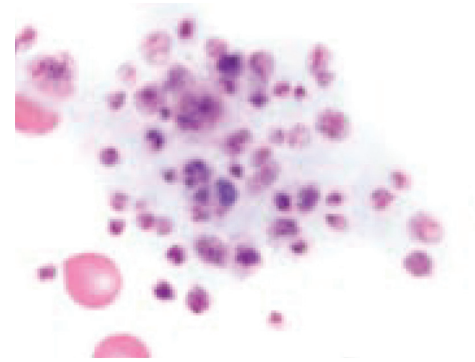


Fig. 2.14D: Platelets in clump

Control of Platelet Formation

Perhaps by a humoral factor called thrombopoietin, acts by a feedback mechanism.

CHAPTER

3

Red Blood Cells

HISTORICAL ASPECTS

Year	*Person*	*Inferance/Finding*
1658	Swammerdam	First observed RBCs, called them "Ruddy globules"
1674	Leeuwenhoek	Described RBCs in human blood
1775	Hewson	Recognised RBCs as flat disks
1851	Funke	Isolated hemoglobin
Later	Hoppe-Seyler	Demonstrated that hemolobin has the property of readily taking up and discharging oxygen.

STRUCTURE

It is one of the most highly specialized cells, consisting of a plasma membrane surrounding a solution of proteins and electrolytes. More than 95% of cytoplasmic protein is the oxygen-transport protein, hemoglobin.

It lacks cytoplasmic organelles like nucleus, mitochondria, or ribosomes. It can neither synthesize new protein nor carry out oxidative reactions associated with mitochondria or undergo mitosis.

Nevertheless, the red cell subserves a number of complex and crucial functions in the human body.

SHAPE

The mature human red blood cell is shaped like a flattened, bilaterally indented sphere, often referred to as a "biconcave disk". On fixed, stained blood smears, it appears circular, with an area of central pallor corresponding to the indented regions, on fixed smears.

The RBC has a diameter of about 7–8 μm. The disk shape is well suited to RBC function. The surface to volume ratio approaches the maximum value in such a shape:

- Facilitates gas transfer
- Biconcave disk is more deformable and can undergo the changes in shape necessary for optimal movement within the microvasculature.
- The plane of the biconcave disk has been found to be oriented in the direction of blood flow. The leading edge becomes pointed and the following edge blunted. In this way, the RBC can negotiate a vessel of about 4 mm diameter.

How does the RBC produce and maintain the resting biconcave shape?

1. Elastic forces within the membrane
2. Surface tension
3. Electrical forces on membrane surface
4. Osmotic and hydrostatic pressures.

The indented region can easily change its location. The RBC is remarkable for its ability to maintain membrane integrity while exhibiting extreme deformability under normal physiologic circumstances. The elasticity of membrane and low viscosity of RBC cytoplasm, permit its movement through very narrow channels within the human body.

Osmotic swelling occurs when the RBC is suspended in hypotonic solutions. First, it becomes cup-shaped and eventually becomes spherical. The cell volume increases but surface area more or less remains the same.

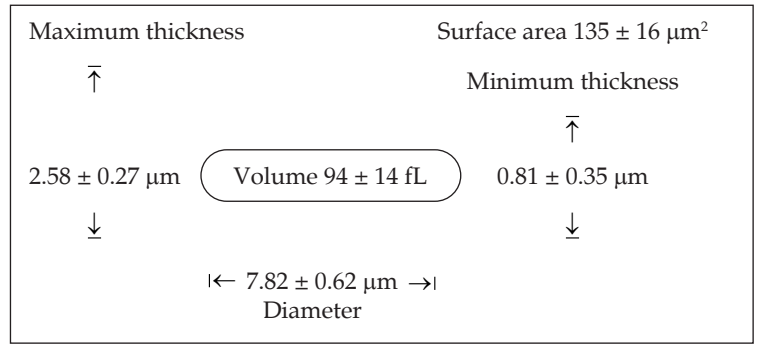
Discocyte to echinocyte (spiculated RBC) transformation takes place when

- Intracellular adenosine triphosphate (ATP) is depleted
- Intracellular calcium is increased
- Cell is exposed to stored plasma, high pH, anionic detergents, lysolecithin or fatty acids
- The cell is placed between a glass (not plastic) slide and coverslip.

Discocyte-stomatocyte transformation occurs when red cells are exposed to low pH, cationic detergents, or phenothiazines.

These cells on fixed smears show a mouth-like "stoma" instead of central pallor, therefore, these cells are called stomatocytes.

Dimensions



PHYSIOLOGY OF THE RED BLOOD CELLS

A normal mature red blood cell has:

- A surface membrane
- Cytoplasm, which is further subdivided into
  - Hemoglobin and
  - Stroma.

RED CELL MEMBRANE (Fig. 3.1)

It is made of 3 layers.

Outer Layer

Hydrophilic layer made of glycoprotein molecules which carry the mucopolysaccharide blood group antigens and absorbed protein.

Middle Layer

It is a hydrophobic phospholipid bilayer stabilized by cholesterol.

Inner Layer

It is a hydrophilic layer containing protein molecules extending toward the cell interior.

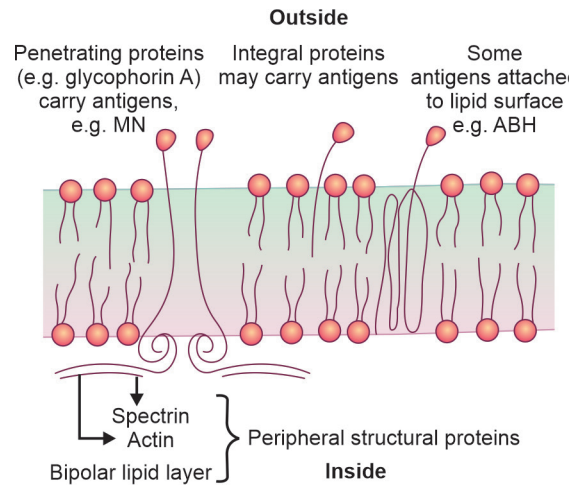


Fig. 3.1: Simplified diagram of lipid and protein structure of the red cell membrane

The red cell surface bears a negatives charge.

- RBC membrane represents a semipermeable lipid bilayer supported by a protein mesh-like cytoskeleton structure. The fluid lipid matrix contains equal amounts of cholesterol and phospholipids with a mosaic of protein interspersed throughout at various intervals.
- The proteins that extend from the outer surface and traverse the entire membrane to the inner cytoplasmic side of the RBC are called "integral" membrane proteins. The "peripheral" proteins are limited to the cytoplasmic surface of the membrane, which is beneath the lipid bilayer and forms the RBC cytoskeleton.
- The protein and lipid layers are organized asymmetrically.
- Chemically, the membrane approximately constitutes 40% lipids, 50% proteins, and 10% carbohydrates.

RED CELL MEMBRANE LIPIDS

Phospholipids

The RBC membrane lipid consists of a bilayer of phospholipids interspersed with molecules of unesterified cholesterol and glycolipids. Two groups of phospholipids are known to possess a distinct asymmetry within the bilayer matrix of the RBC: Choline phospholipids and amino phospholipids.

- Choline phospholipids, consisting of phosphatidylcholine and sphingomyelin, are primarily located on the outer half of the lipid bilayer readily accessible to the external environment. There is an energy dependent fatty acid exchange between plasma fatty acids and the RBC membrane. Therefore changes in body lipid transport and metabolism may cause abnormalities in the plasma phospholipid concentration, thereby, resulting in a decreased RBC survival in circulation.
- Amino phospholipids, in contrast, consisting of phosphatidylethanolamine and phosphatidylserine, are located almost exclusively on the inside half or cytoplasmic side of the RBC membrane along with phasphatidylinositol. This specific orientation of these phospholipids maintains a precise lipid pattern that is critical to normal RBC survival in circulation. Alteration of this arrangement leading to the abnormal appearance of these amino phospholipids on the outer surface of the lipid bilayer promotes activation of the clotting cascade and may result in extravascular hemolysis. Stabilization of this phospholipid asymmetry in the RBC membrane is maintained through the interaction of specific peripheral proteins.

Glycolipids and Cholesterol

- The majority of the glycolipids are located in the outer half of the lipid bilayer and interact with glycoproteins to form many of the RBC antigens.
- Cholesterol is approximately equally distributed, being located on both sides of the lipid bilayer inserted between the choline and amino phospholipids. Cholesterol comprises 25% of the RBC membrane lipids and is present in a 1:1 molar ratio with phospholipids. RBC cholesterol is in continual exchange with plasma cholesterol and is therefore affected by changes in body lipid transport.
- Increased cholesterol accumulation in the RBC produces target cells, and may cause RBC membrane damage. Acanthocytes or spiny RBCs are also associated with excess cholesterol accumulation on RBC membrane (as may occur in lipid disorders, such as abetalipoproteinemia). Excess cholesterol makes the cell less deformable and reduces their life span. LCAT deficiency (lecithin cholesterol acyltransferase) leads to excessive free cholesterol in both plasma and RBC membrane, resulting in, among other problems, a chronic hemolytic anemia.

RBC Membrane Proteins (Fig. 3.2)

It is estimated that 10 major and 200 minor proteins are asymmetrically organized within the RBC membrane. After solubilization of the RBC membrane with the detergent sodium dodecyl sulfite (SDS), membrane proteins can be separated by polyacrylamide gel electrophoresis and stained. The separated RBC membrane proteins are numbered 1–8 when stained with Coomassie blue and 1–4 when stained with periodic acid-Schiff (PAS) stain, which is the basis of their nomenclature. The proteins range in molecular weight from 16,000 to 240,000 daltons. Two of the most important constituents include glycophorin an "integral membrane protein and spectrin, a "peripheral" membrane protein.

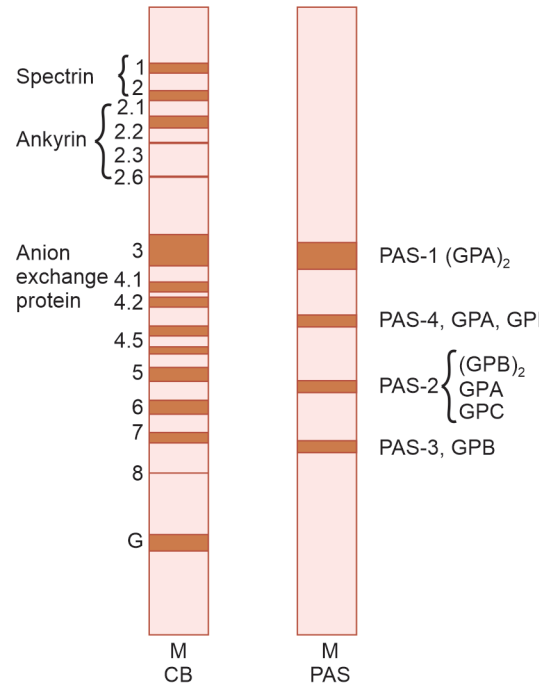


Fig. 3.2: Schematic illustration of SDS polyacrylamide gel electrophoresis patterns of red cell membrane proteins stained with Coomassie blue (CB) and sialoglycoproteins stained with PAS (periodic acid-Schiff) stain. GPA, GPB and GPC refer to glycophorins A, B, and C respectively. $(GPA)_2$ and $(GPB)_2$ are the dimers and GPA-GPB is the heterodimer of GPA-GPB

Integral Membrane Proteins

Glycophorin, the major integral membrane protein, is the principal RBC glycoprotein, representing 10% of the total membrane protein. The molecule contains approximately 60% carbohydrate and accounts for most of the membrane sialic acid, which gives the RBC and its negative charge. Glycophorin spans the entire thickness of the lipid bilayer and appears on the external surface of the RBC membrane, accounting for the location of RBC antigens.

The majority of these proteins, as mentioned previously, carry RBC antigens and are receptors (such as glycophorins) or are transport proteins (such as band 3, the anion exchange channel glycoprotein). It is thought that band 3 and glycophorins play a major role in anchoring the RBC membrane cytoskeleton to the lipid bilayer. As a result, lateral motility of these integral proteins within the lipid bilayer is relatively restricted.

Peripheral Proteins

Spectrin, the principal "Peripheral" protein, is a large molecule that represents approximately 75% of the peripheral protein and 25% of the total membrane protein. Spectrin is intimately related to RBC membrane integrity as it binds with other peripheral proteins such as actin (band 5), ankyrin (band 2.1), and band 4.1 to form a "skeleton" of microfilaments on the inner surface of RBC membrane. These microfilaments strengthen the membrane, protecting the cell from being broken by circulatory shear forces, and also control the biconcave shape and deformability of the cell.

The normal chemical composition as well as structural arrangement and molecular interactions of the RBC membrane are crucial to the normal red cell survival in circulation of 120 days. In addition, they play a critical role in two important RBC characteristics: Deformability and permeability.

Deformability

Loss of ATP (energy) levels leads to a decrease in the phosphorylation spectrin and in turn, a loss of membrane deformability. An accumulation or increase in deposition of membrane calcium also increases the membrane rigidity and loss of pliability. These cells are at a marked disadvantage when they pass through the small (3–5 μm diameter) orifices of the spleen, one of whose functions is extravascular sequestration and removal of aged, damaged, or less deformable RBCs or fragments of their membrane. The loss of RBC membrane is exemplified by the formation of spherocytes, cells with a reduced surface-to-volume ratio, and "bite cells" in which the removal of a portion of membrane has left a permanent indentation in the remaining cell membrane. The survival of these forms is also shortened.

Permeability

The RBC membrane is freely permeable to water and anions, chloride (Cl^-) and bicarbonate (HCO_3^-) traverse the membrane in less than a second. It is thought that the massive exchange of HCO_3^- and Cl^- ions occurs through a large numbers of exchange channels formed by the integral protein, band 3, a glycoprotein described earlier. In contrast the RBC membrane is relatively impermeable to cations with a half-time exchange of sodium (Na^+) and potassium (K^+) of over 30 hours. It is primarily through the control of the sodium and potassium intracellular levels that the RBC maintains its volume and water homeostasis. The erythrocyte intracellular:extracellular ratios of sodium and potassium are 1:12 and 25:1, respectively. The passive influx of sodium and potassium is controlled by as many as 300 cationic pumps, which actively transport sodium out of the cell and potassium into the cell. These pumps are energy dependent requiring ATP, and membrane enzyme sodium-potassium ATPase. The full activation of the sodium-potassium ATPase pumps requires the presence of RBC membrane aminophospholipid, phosphatidylserine. Similarly, calcium (Ca^{++}) is also actively pumped from the interior of the RBC through the energy-dependent calcium ATPase cationic pump. Calmodulin, a cytoplasmic calcium-binding protein, is

speculated to control these calcium-ATPase pumps, preventing excessive intracellular calcium buildup, which is harmful to the RBC, resulting in shape changes and loss of deformability. The permeability properties of the RBC membrane, as well as active cation transport, are crucial to preventing colloid osmotic hemolysis and controlling the volume of the red cell. In addition, ATP-depleted cells allow the accumulation of excess intracellular calcium and sodium, followed by potassium and water loss, resulting in dehydrated, rigid cell later sequestered by spleen. The energy required by all those pumps is provided by ATP.

HEMOGLOBIN STRUCTURE AND FUNCTION

Hemoglobin is a conjugated protein with a molecular weight of approximately 68,000, comprises 95% of the RBC's dry weight, or 33% of the RBC's weight by volume. Approximately 65% of the hemoglobin occurs during the nucleated stages of RBC maturation, and 35% occurs during the reticulocyte stage. Normal hemoglobin consists of globin (a tetramer of two pairs of polypeptide chains) and four heme groups, each of which contains a protoporphyrin ring plus iron (Fe^{++}).

Hemoglobin Synthesis

Normal hemoglobin production is dependent on three processes:
1. Adequate iron delivery and supply
2. Adequate synthesis of protoporphyrins (the precursor of heme)
3. Adequate globin synthesis.

Iron Delivery and Supply

Iron is delivered to the membrane of the RBC precursor (say a reticulocyte) by the protein carrier transferrin. The majority of the iron that crosses the membrane and enters the cytoplasm of the cell is committed to hemoglobin synthesis and thereby proceeds to mitochondria for insertion into the protoporphyrin ring to form heme. Excess iron in the cytoplasm aggregates as ferritin, the amount of which is dependent on the ratio between the level of plasma iron and the amount of iron required by the erythrocyte for hemoglobin synthesis. Two-thirds of the total body iron supply is bound to heme in the hemoglobin (Fig. 3.3).

Synthesis of Protoporphyrins

Protoporphyrin synthesis begins in the mitochondria with the formation of delta aminolevulinic acid (δ-ALA) from glycine and succinyl-CoA, which is the major rate limiting step in heme biosynthesis, the mitochondrial enzyme δ-ALA synthetase, which mediates this reaction is influenced by erythropoietin and requires the presence of the cofactor pyridoxal phosphate (vitamin B_6).

In the cytoplasm, condensation of two molecules of δ-ALA, catalyzed by δ-ALA dehydrase, produces the pyrrole porphobilinogen (PBG). Because four

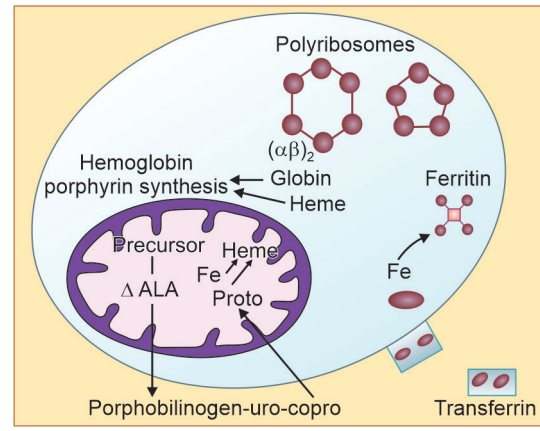


Fig. 3.3: Synthesis of hemoglobin in a reticulocyte

molecules are involved in the reaction, the formation of four types of isomers is theoretically possible. However, only two types of isomers have occurred physiologically UPG—I and III—and virtually all the UPG is the type III isomer. Coproporphyrinogen (CPG) is next formed via decarboxylation reactions from UPG III. The final steps of heme synthesis are carried out in the mitochondria and involve the formation of protoporphyrinogen (PP) from CPG III. Because PP chemically has three types of side chains, 15 possible isomers of PP can form, compared with four possible UPG and CPG isomers. However, normal mitochondrial physiology leads to the formation of only one of these isomers, PP IX, from CPG III. After further conversion of protoporphyrin IX, the incorporation of iron results in heme (ferroprotoporphyrin IX) formation.

Porphyrinogens, not porphyrins, are the intermediates of heme synthesis. Porphyrinogens are unstable tetrapyrroles that are readily and irreversibly oxidized to form porphyrins. In contrast, porphyrins are highly stable resonating molecules that are normally found in small quantities in the urine as a result of normal RBC catabolism.

Globin Synthesis

Globin chain synthesis occurs on RBC-specific cytoplasmic ribosomes, which are initiated from the inheritance of various structural genes. Each gene results in the formation of a specific polypeptide chain. Each somatic cell, including the RBC, contains four alpha (α) genes, two beta (β) genes, two delta (δ) genes, and four gamma (γ) genes. The alpha genes are located on chromosome 16, and the beta, delta and gamma genes are located on chromosome 11. The resulting products are called alpha, beta, delta and gamma chains. In the fetus, it should be remembered that during the first few weeks of life, a different polypeptide chain, epsilon (ε), is synthesized forming tetramer molecules that are designated "embryonic" hemoglobin (ε_4 or $\alpha_2\varepsilon_2$).

All adult normal hemoglobins are formed as tetramers consisting of two alpha chains. plus two (non-alpha) globin chains. Normal adult RBCs contain the following types of hemoglobins:

- Ninety two to ninety five percent of the hemoglobin is HbA, consisting of $\alpha_2\,\beta_2$ chains.
- Three to five percent of the hemoglobin is HbA1c (glycosylated), which consists of α_2 $(\beta\text{-NH-glucose})_2$ chains
- Two to three percent of the hemoglobin is HbA_2, consisting of $\alpha_2\,\delta_2$ chains.
- One to two percent of the hemoglobin is (fetal) HbF, consisting of $\alpha_2\,\gamma_2$ chains.

Each synthesized globin chain links with heme (ferroprotoporphyrin IX) to form hemoglobin, which primarily consists of two alpha chains, two beta chains, and four heme groups. Normal alpha chains consist of 141 amino acids, while normal beta chains consist of 146 amino acid residues. The precise order of amino acids is critical to the hemoglobin molecule's structure and function. The substitution of even one amino acid, such as valine, for the normal glutamic acid at the sixth position on the β chain, can result in such functional abnormalities as a hemoglobin that tends to polymerize when deoxygenated (sickle hemoglobin, HbS). An adequate amount of β-chain synthesis is also important since decreased production of one of the polypeptide chains leads to a group of disorders known as thalassemias, the more common forms refer to decrease in β-chain production and α-thalassemia refers to a decrease in α-chain production.

The rate of globin chain synthesis is directly related to the rate of porphyrin synthesis, and vice versa; protoporphyrin synthesis is reduced when globin synthesis is impaired. There is, however, no such relationship with iron uptake when either globin or protoporphyrin synthesis is impaired; iron accumulates in the RBC cytoplasm as ferritin aggregates.

(The iron-ladden nucleated RBC is termed a "sideroblast" and anucleated form a "siderocyte".) (Iron can be stained with Prussian blue stain).

HEMOGLOBIN FUNCTION

The primary function of hemoglobin is gas transport (oxygen and carbon dioxide): Delivery and release of oxygen to tissues and facilitation of carbon dioxide excretion. Owing to hemoglobin's multichain structure, the molecule is capable of a considerable amount of allosteric movement as it loads and unloads oxygen. One of the most important controls of hemoglobin affinity for oxygen is the RBC organic phosphate 2, 3-diphosphoglycerate (2, 3-DPG). The unloading of oxygen by hemoglobin is accompanied by the widening of the space between beta chains and the binding of 2,3-DPG, on a mole-for-mole basis, with the formation of anionic salt ridges between the beta chains. The resulting confirmation of the deoxyhemoglobin molecule is know as the tense ("T") form, which has a lower affinity for oxygen. When hemoglobin loads oxygen and becomes oxyhemoglobin, the established salt bridges are broken and beta chains are pulled together, expelling, the 2, 3-DPG. This is the relaxed ("R") form of hemoglobin molecule and has a higher affinity for oxygen (Fig. 3.4).

These allosteric changes that occur as the hemoglobin loads and unloads oxygen are referred to as the "respiratory movement". The dissociation and the binding of oxygen by hemoglobin are not directly proportional to the PO_2 of its environment but instead exhibit a sigmoid–curve relationship, the hemoglobin–oxygen dissociation curve is depicted in Fig. 3.5.

The shape of this curve is very important physiologically, as it permits a considerable amount of oxygen to be delivered to the tissues with a small drop in oxygen tension. For example, in the environment of lungs, where the partial pressure of oxygen (oxygen tension), measured in millimeters of mercury (Hg), is nearly 100 mm Hg, the hemoglobin molecule is almsot 100% saturated with oxygen (point A, as given in Fig. 3.5). As the RBC travels to tissues where the partial pressure of oxygen drops to an average 40 mm Hg (mean venous oxygen tension), the hemoglobin saturation drops to approximately 75% saturation, releasing about 25% of oxygen to tissues (point B).

In situations such as hypoxia, a compensatory "shift to the right" of hemoglobin–oxygen dissociation curve occurs to alleviate a tissue oxygen deficit (Fig. 3.6).

This rightward shift of the curve, mediated by increased levels of 2,3-DPG, results in a decrease in hemoglobin's affinity for the oxygen molecule and an increase in oxygen delivery to tissues. Note

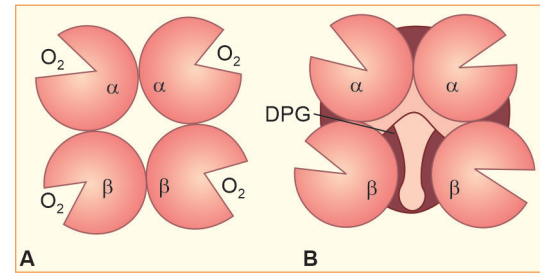


Fig. 3.4: (A) Oxyhemoglobin; (B) Deoxyhemoglobin

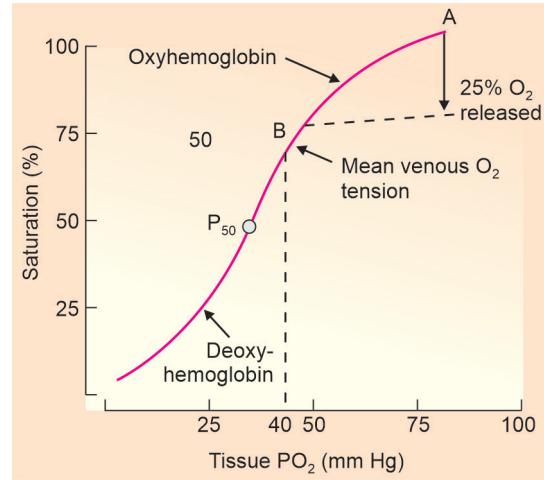


Fig. 3.5: Normal hemoglobin–oxygen dissociation curve

that the oxygen saturation of hemoglobin in the environment of the tissues [40 mm Hg PO_2 (see point in the Fig. 3.6)] is now only 50%; the other 50% of the oxygen is being released to the tissues. The RBCs thus have become more efficient in terms of oxygen delivery.

Therefore, a patient who is suffering from anemia due to loss of RBCs may be able to compensate by shifting the oxygen dissociation curve to the right, making the RBCs, while few in number, more efficient. Some patients may be able to tolerate anemia better than others because of this compensatory mechanism. A shift to the right may also occur in response to acidosis or a rise in body temperature. This shift to the right of the hemoglobin-oxygen dissociation curve is only one way in which patients may compensate for various types of hypoxia. Other ways include an increase in total cardiac output and increase in erythropoiesis.

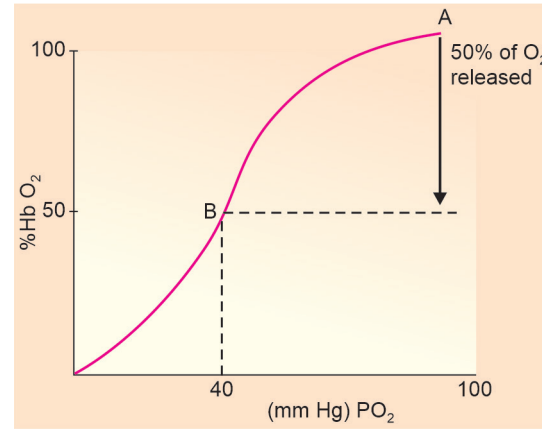


Fig. 3.6: Right-shifted hemoglobin–oxygen dissociation curve

A "shift to the left" of the hemoglobin–oxygen dissociation curve results, conversely, in an increase in hemoglobin-oxygen affinity and decrease in oxygen delivery to tissues (Fig. 3.7).

With such a dissociation curve RBCs are much less efficient since only 12% of oxygen can be released to the tissue (point B). Among conditions that can shift the oxygen dissociation curve to the left are alkalosis; increased quantities of abnormal hemoglobins, such as methemoglobin and carboxyhemoglobin; increased quantities of HbF; or multiple transfusions of 2, 3-DPG-depleted stored blood.

Active Red Cell Metabolic Pathways (Maintenance of Hemoglobin Function)

Active RBC metabolic pathways are necessary for the production of adequate ATP levels. Energy so generated is crucial to RBC survival and function in that it is necessary for maintaining:

1. Hemoglobin function
2. Membrane integrity and deformability
3. RBC volume, and
4. Adequate amounts of reduced pyridine nucleotides.

RBCs generate energy almost exclusively through the anerobic breakdown of glucose since the metabolism of the anucleated RBC is more limited than that of other body cells. The adult RBC possesses little ability to metabolize fatty and amino acids. Additionally, mature RBCs contain no mitochondrial apparatus for oxidative metabolism.

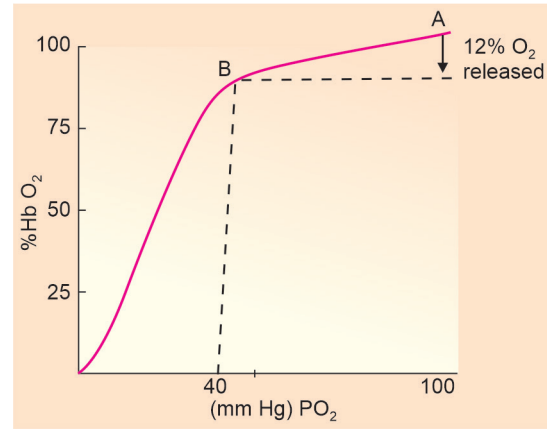


Fig. 3.7: Left-shifted hemoglobin–oxygen dissociation curve

Table 3.1: Metabolic activities during various stages of RBC maturation

	Nucleated	*Reticulocyte*	*Mature RBC*
Replication	+	–	–
DNA synthesis	+	–	–
RNA synthesis	+	–	–
Lipid synthesis	+	+	–
RNA presence	+	+	–
Heme synthesis	+	+	–
Protein synthesis	+	+	–
Mitochondria	+	+	–
Krebs' TCA cycle	+	+	–
EM pathway	+	+	+
Pentose phosphate shunt	+	+	+
Maturation and/or senescence	+	+	+

Fortunately enough, RBCs metabolic pathways are anerobic, since its function is to deliver oxygen and not to consume it. Four pathways of RBC metabolism will be considered: The anaerobic glycolytic pathway and three ancillary pathways that serve to maintain the function of hemoglobin. All of these processes are essential if the RBC is to transport oxygen and maintain the physical characteristics required for its survival in circulation (Table 3.1).

Ninty percent of the ATP needed by RBCs are generated by the Embden–Meyerhof glycolytic pathway, the RBCs main metabolic pathway. Here the metabolism of glucose results in the net generation of two molecules of ATP. Although this ATP synthesis is inefficient when compared with cells that use the Krebs' cycle (aerobic mechanism), it provides sufficient ATP for the RBC's requirements. Glycolysis also generates NADH from NAD^+, important in some of the RBCs other metabolic pathways (Fig. 3.8).

Another 5–10% of glucose is metabolized by the hexose monophosphate shunt (also called the phosphogluconate pathway), which produces the pyridine nucleotide NAPH from $NADP^+$. NADPH, together with reduced glutathione, provides the main line of defence for the RBC against oxidative injury. Oxidative drugs as well as infections, can cause accumulation of hydrogen peroxide and other oxidants, which can be toxic to cell proteins (Fig. 3.8).

When the hexose monophosphate pathway is functionally deficient, the amount of reduced glutathione becomes insufficient to neutralize intracellular oxidants. This results in globin chain denaturation and precipitation as aggregates (Heinz bodies) within the cell. If this process sufficiently damages the membrane, cell destruction occurs. Inherited defects in the pentose phosphate glutathione pathway, the most common of which is G6PD deficiency, result in the formation of Heinz bodies with subsequent extravascular hemolysis. (It should be remembered that glutathione not only is crucial to keeping hemoglobin in a functional state but also is important in maintaining RBC integrity by reducing sulfhydryl groups of hemoglobin, membrane protein, and enzymes subsequent to oxidation).

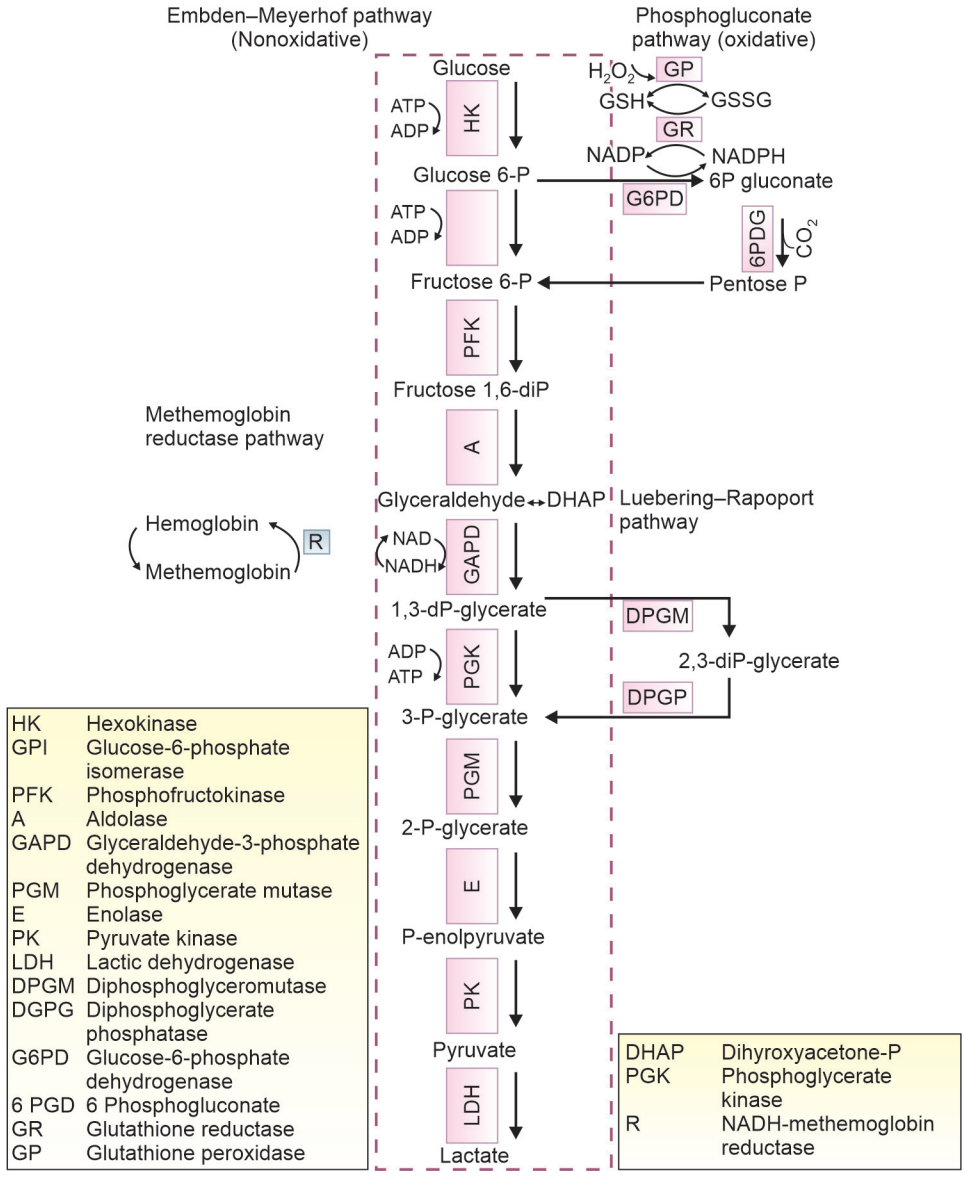


Fig. 3.8: Red cell metabolism

Reactions within RBC to prevent accumulation of oxidants

Reaction A RBC+ infection or oxidant $\longrightarrow H_2O_2$

Reaction B $H_2O_2 + 2GSH$ (reduced glutathione) $\xrightarrow[\text{peroxidase}]{\text{Glutathione}} GSSG + 2H_2O$ oxidized glutathione

Reaction C $GSSG + NADPH$ (reduced form) $+ H^+ \xrightarrow{\text{Glutathione reductase}} 2GSH + NADP^+$ (oxidized form)

Reaction D G-6-P (Glucose-6-phosphate) $+ NADP^+ \xrightarrow[\text{dehydrogenase}]{\text{Glucose-6-phosphate}} 6PG + NAPH + H^+$ (6-phospho-gluconate)

The methemoglobin reductase pathway is another important component of RBC metabolism. Two methemoglobin reductase systems are important in maintaining heme iron in the reduced (Fe^{++}, ferous), functional state. Both pathways are dependent on the regeneration of reduced pyridine nucleotides and are referred to as the NADH and NADPH methemoglobin reductase pathways. In the absence of the enzyme methemoglobin reductase and the reducing action of the pyridine nucleotide NADH, there is an accumulation of methemoglobin resulting from the conversion of the ferrous iron of heme to the ferric form (Fe^{+++}). Methemoglobin is a nonfunctional form of hemoglobin, having lost its oxygen transport capability as the metheme portion cannot combine with oxygen. Normal efficiency of the methemoglobin reductase pathway is exemplified by the fact that usually no more than 1% of RBC hemoglobin exists as methemoglobin in the RBCs of healthy persons (Fig. 3.8).

Another important pathway which is important to RBC function is the Leubering–Rapoport shunt. This pathway causes an extraordinary accumulation of the RBC organic phosphate 2,3-DPG, important because of its profound effect on hemoglobin's affinity for oxygen and also because its stores can serve as a reserve for additional ATP generation (Fig. 3.8).

RBC Senescence/Aging

RBC, a 7 μm biconcave disc, travels approximately 200–300 miles during its 120 days or so life span.

During its life span RBCs undergo the process of aging just as other cells of the human body. Each day 1% of the old RBCs in circulation are extricated by a system of fixed macrophages in the body known as the reticuloendothelial system (RES). These RBCs are replenished by 1% of the younger RBCs, reticulocytes, from the bone marrow storage pool. As RBCs age, certain glycolytic enzymes decrease in activity, resulting in a decrease in the production of energy and loss of deformability. At a certain point the RBCs are no longer able to traverse the microvasculature and are phagocytosed by the RES cells (Table 3.2). Although RES cells are located in various organs and throughout the body, those of the spleen, called "littoral cells" are the most sensitive detectors of RBC abnormalities. This is mainly due to the typical microvasculature of spleen which reduces the plasma volume and flow of blood, exposing the RBCs to the phagocytic action of RES cells of the spleen for a longer time. 90% of the destruction of aged RBCs occurs by the process of extravascular

Table 3.2: Changes occurring during aging of RBC

Increases	*Decreases*
Membrane bound IgG	Several enzyme activities
Density	Sialic acid
Spheroidal shape	Deformability
MCHC	MCV
Internal viscosity	Phospholipid
Agglutinability	Cholesterol
Na^+	K^+
Methemoglobin	Protoporphyrin
Oxygen affinity	

hemolysis. During this process, old or damaged RBCs are phagocytized by the RES cells and digested by their lysosomes. The hemoglobin molecule is disassembled, being broken down into its various components. The iron recovered is salvaged and returned to plasma protein carrier, transferrin, to the erythroid precursors in the marrow for synthesis of the new hemoglobin. Globin is broken down into amino acids and sent over to the amino acid pool of the body. Finally, the photoporphyrin ring of heme is broken down, exhaling its alpha carbon as carbon monoxide. The opened tetrapyrrole, biliverdin is converted to bilirubin and carried by plasma protein albumin to the liver. In the liver, bilirubin is conjugated to bilirubin glucuronide and excreted along with bile into intestines. By bacterial action, in the intestines, it is converted into urobilinogen (stercobilinogen) and excreted via the stool. A small amount of urobilinogen is reabsorbed through the enterohepatic circulation, filtered by kidneys, and excreted in small amounts in the urine.

Five to ten percent of normal RBC destruction occur through the process of intravascular hemolysis. Here, the RBC breakdown occurs in the lumen of the blood vessels. The RBC ruptures, releasing hemoglobin directly into the bloodstream (Fig. 3.9).

The hemoglobin molecule dissociates into alpha-beta dimers and is picked up by the protein carrier, "haptoglobin". The haptoglobin–hemoglobin complex prevents renal excretion of the hemoglobin and carries the dimers to the liver cell for further catabolism. The hepatocyte processing and uptake is identical at this point to the process earlier described for extravascular hemolysis. Haptoglobin levels, therefore, fall in plasma as it is removed as the hemoglobin–haptoglobin complex. It is thought that as little as 1–2 mL of RBC intravascular hemolysis can totally deplete the amount of plasma haptoglobin. Normally, 50–200 mg/dL of plasma haptoglobin is available and represents the hemoglobin-dimer binding capacity. As haptoglobin is depleted, unbound hemoglobin dimers appear in plasma (hemoglobinemia) and are filtered through the kidneys and reabsorbed by the renal tubular cells. The renal tubular uptake capacity is approximately 5 g per day of filtered hemoglobin. Beyond this level, free hemoglobin appears in urine (hemoglobinuria) (Fig. 3.10).

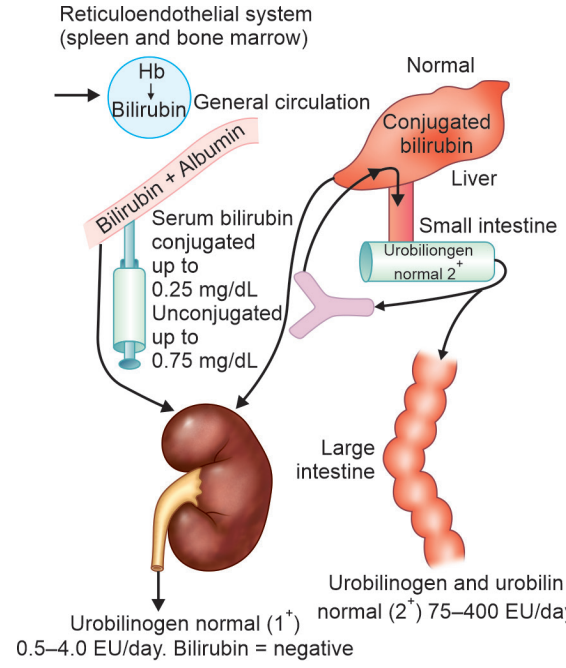


Fig. 3.9: Normal extravascular hemolysis

Hemoglobinuria is always associated with hemoglobinemia. Normal plasma hemoglobin levels are 2–5 mg/dL, which is exceeded when free hemoglobin is released as a result of excessive intravascular hemolysis. Depending upon the amount of hemolysis and type of hemoglobin, the plasma may be pink, red, or brown. Likewise in hemoglobinuria the urine may also be pink, red, brown or black. Two hemoglobin pigments, oxyhemoglobin and methemoglobin, are produced by auto-oxidation of the hemoglobin in the urinary tract when the urine is acidic. Oxyhemoglobin is bright red, and methemoglobin is dark brown. The color of

urine, therefore, depends upon the amount of hemolysis and concentration and relative proportions of these two hemoglobins. Oxyhemoglobin predominates in alkaline urine, and methemoglobin predominates in acidic urine.

Hemoglobin, which is neither processed by the kidneys nor bound to haptoglobin, is oxidized to methemoglobin, which is further disassembled as metheme groups are released and globin degraded. Free metheme is quickly bound by another transport protein "hemopexin", and carried to the liver cell to be catabolized as described earlier. The heme binding capacity of hemopexin is approximately 50–100 mg/dL, and when this is exceeded the metheme groups combine with albumin to form methemalbumin. Albumin cannot transfer the metheme across the hepatocytic membrane for further degradation. As a result, the methemalbumin circulates until additional hemopexin is produced by the liver to serve as the protein carrier. It is this circulating methemalbumin that imparts a brown tinge to the plasma or blood. Under normal circumstances, the intravascular hemolysis is so minimal that it is limited to the involvement of only haptoglobin, which is rarely depleted. Other processes, described herein, come into play, only when excessive intravascular hemolysis occurs, which occurs in various types of hemolytic anemias.

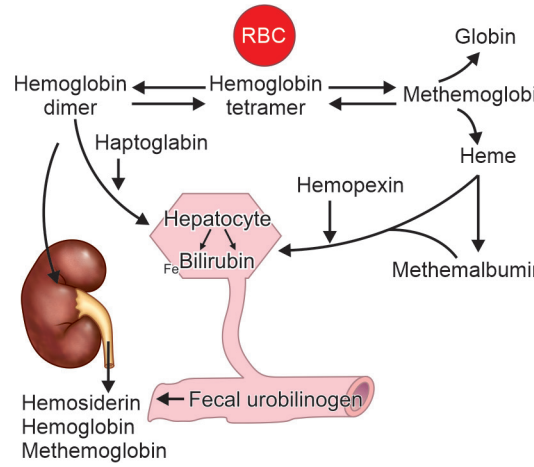


Fig. 3.10: Intravascular hemolysis

Nutritional and Other Requirements of a Red Cell

For normal hemopoiesis the following substances are required, deficiency of these may lead to anemia.

- Amino acids: For synthesis of globin of hemoglobin.
- Iron: Needed for heme synthesis.
- Vitamin B_{12} and folate essential for nucleic acid synthesis, mainly DNA.
- Vitamin C: Plays a part in folate metabolism.
- The B vitamins: are needed for a normal hemopoiesis. The more important ones are riboflavin, pyridoxine, nicotinic acid and pantothenic acid.
- Vitamin E: Supposed to be important for hemopoiesis in infants only.
- Trace metals, e.g. copper and cobalt. Copper appear to be catalyst in hemoglobin formation and cobalt is an essential constituent of vitamin B_{12}.

NORMAL RED CELL VALUES

There is considerable physiologic variation in red cell values of normal subjects and it depends upon:

- The age and sex of the individual.
- Diurnal and day-to-day fluctuations.

RBC COUNT

Age/Sex/Phase	*Conventional units*	*SI units*
Adult females	4.0–5.5 million/μL	4.0–5.5 $\times 10^{12}$/L
Pregnant		
Trimester 1	4.0–5.0 million/μL	4.0–5.0 $\times 10^{12}$/L
Trimester 2	3.2–4.5 million/μL	3.2–4.5 $\times 10^{12}$/L
Trimester 3	3.0–4.9 million/μL	3.0–4.9 $\times 10^{12}$/L
Postpartum	3.2–5.0 million/μL	3.2–5.0 $\times 10^{12}$/L
Adult males	4.5–6.2 million/μL	4.5–6.2 $\times 10^{12}$/L
Children		
Newborn		
Day 1	4.1–6.1 million/μL	4.1–6.1 $\times 10^{12}$/L
Days 2–8	Average 5.1 million/μL	5.1 $\times 10^{12}$/L
Days 9–13	Average 5.0 million/μL	5.0 $\times 10^{12}$/L
2–8 weeks	3.8-5.6 million/μL	3.8–5.6 $\times 10^{12}$/L
3–5 months	3.8–5.2 million/μL	3.8–5.2 $\times 10^{12}$/L
6–11 months	Average 4.6 million/μL	4.6 $\times 10^{12}$/L
1–2 years	3.6–5.2 million/μL	3.6–5.5 $\times 10^{12}$/L
3 years	Average 4.5 million/μL	4.5 $\times 10^{12}$/L
4 years	4.0–5.2 million/μL	4.0-5.2 $\times 10^{12}$/L
5 years	Average 4.6 million/μL	4.6 $\times 10^{12}$/L
6–10 years	Average 4.7 million/μL	4.7 $\times 10^{12}$/L
11–15 years	Average 4.8 million/μL	4.8 $\times 10^{12}$/L

Hemoglobin

Adult females	12–16 g/dL (g%)	7.4–9.9 mmol/L
Pregnant		
Trimester 1	11.4–15.0 g/dL (g%)	7.1–9.3 mmol/L
Trimester 2	10.0–14.3 g/dL (g%)	6.2–8.9 mmol/L
Trimester 3	10.2–14.4 g/dL (g%)	6.3–8.9 mmol/L
Postpartum	10.4–15.0 g/dL (g%)	6.4–9.3 mmol/L
Adult males	14.0–18.0 g/dL (g%)	8.7–11.2 mmol/L
Children		
Newborn		

Day 1	15.5–24.5 g/dL (g%)	9.6–15.2 mmol/L
Days 2–3	Average 19.0 g/dL (g%)	11.8 mmol/L
Days 4–8	14.3–22.3 g/dL (g%)	8.9–13.8 mmol/L
Days 9–13	Average 16.5 g/dL (g%)	10.2 mmol/L
2–8 weeks	10.7–17.3 g/dL (g%)	6.6–10.7 mmol/L
3–5 months	9.9–15.5 g/dL (g%)	6.1–9.6 mmol/L
6–11 months	Average 11.8 g/dL (g%)	7.3 mmol/L
1–2 years	9.0–14.6 g/dL (g%)	5.6–9.0 mmol/L
3–9 years	9.4–15.5 g/dL (g%)	5.8–9.6 mmol/L
10 years	10.7–15.5 g/dL (g%)	6.6–9.6 mmol/L
11–15 years	13.4 g/dL (g%)	8.3 mmol/L
Panic low level	<5	<3.1
Panic high level	>18	>11.2

Hematocrit (Packed cell volume) (PCV)

Adult females	37–47%	0.37–0.47 L/L
Pregnant		
Trimester 1	35–46%	0.35–0.46 L/L
Trimester 2	30–42%	0.30–0.42 L/L
Trimester 3	34–44%	0.34–0.44 L/L
Postpartum	34–44%	0.34–0.44 L/L
Adult males	40–54%	0.40–0.54 L/L
Children		
Newborn	42–68%	0.42–0.68 L/L
3 months	29–54%	0.29–0.54 L/L
1 year	29–41%	0.29–0.41 L/L
3 years	31–44%	0.31–0.44 L/L
10 years	34–45%	

Mean Cell (corpuscular) Volume (MCV)

Adults	76–96 μ^3	76–96 fL
Children		
Newborn		
Day 1	106 μ^3	106 fL
Days 2–3	105 μ^3	105 fL
Days 4–8	103 μ^3	103 fL
Days 9–13	98 μ^3	98 fL

2–8 weeks	90 μ^3	90 fL
3 months	82 μ^3	82 fL
4–5 months	80 μ^3	80 fL
6–11 months	77 μ^3	77 fL
1 year	78 μ^3	78 fL
2 years	77 μ^3	77 fL
3 years	79 μ^3	79 fL
4–10 years	80 μ^3	80 fL
11–15 years	82 μ^3	82 "fL

Mean Cell Hemoglobin (MCH)

Adults	26–34 pg	1.61–2.11 fmol
Children		
Newborn		
Day 1	Average 38 pg	2.36 fmol
Days 2–3	Average 37 pg	2.30 fmol
Days 4–8	Average 36 pg	2.23 fmol
Days 9–13	Average 33 pg	2.05 fmol
2–8 weeks	Average 30 pg	1.86 fmol
3 months	Average 28 pg	1.73 fmol
4–5 months	Average 27 pg	1.67 fmol
6–11 months	Average 26 pg	1.61 fmol
1–2 years	Average 25 pg	1.55 fmol
3 years	Average 26 pg	1.61 fmol
4–10 years	Average 27 pg	1.67 fmol
11–15 years	Average 28 pg	1.73 fmol

Mean Cell Hemoglobin Concentration (MCHC)

Adults	31–36%	19.2–22.34 mmol/L
Children	31–38%	19.2–23.58 mmol/L
Newborn		
Day 1	Average 36%	22.34 mmol/L
Days 2–8	Average 35%	21.72 mmol/L
Days 9–13	Average 34%	21.10 mmol/L
2–8 weeks	Average 33%	20.48 mmol/L
3–5 months	Average 34%	21.10 mmol/L

6–11 months	Average 33%	20.48 mmol/L
1–2 years	Average 32%	19.86 mmol/L
3 years	Average 35%	21.72 mmol/L
4–15 years	Average 34%	21.10 mmol/L

Reticulocyte Count Blood

Adult females	0.5–2.5%	$0.005–0.025 \times 10^{-3}$
Adult males	0.5–1.5%	$0.005–0.015 \times 10^{-3}$
Cord blood	3.0–7.0%	$0.030–0.070 \times 10^{-3}$
New born	1.1–4.5%	$0.011–0.045 \times 10^{-3}$
Infants	0.5–3.1%	$0.005–0.031 \times 10^{-3}$
Children >6 months	0.5–4.0%	$0.005–0.040 \times 10^{-3}$

Mean Cell Diameter (Erythrocyte: Dry film)

Adult 6.7–7.7 μm

Diurnal and Day-to-day Variation

Hemoglobin values are:
- Highest in the morning
- Lowest in evening.

(A change in the Hb must be 1.5 g% or more to be considered definitely significant).

Red Cell Absolute Values/Indices

These can be calculated from:
a. Hematocrit (PCV)
b. Hemoglobin estimation, and
c. Red cell count.

Mean Cell Volume (MCV)

$$\text{MCV} = \frac{\text{Packed cell volume}}{\text{Red cell count pet liter}} \times 10^{15}\,\text{fL}$$

Mean Cell Hemoglobin (MCH)

$$\text{MCH} = \frac{\text{Hemoglobin in g\%}}{\text{Red cell count pet liter}} \times 10^{13}\,\text{pg}$$

Mean Cell Hemoglobin Concentration (MCHC)

$$\text{MCHC} = \frac{\text{Hemoglobin in \%}}{\text{Packed cell volume}} \times \text{g\%}$$

ANEMIA

DEFINITION

It is defined as a reduction is the concentration of hemoglobin in the peripheral blood below the normal for the age and sex of the patient.

Broadly speaking the levels for adult males ia 14 g% and adult females is 12 g%, of late these values have been brought down to 13.2 g% for adult males and 11.7 g% for adult females respectively. The lower limit for both male and female children from three months to puberty is considered to be 10.7 g%, it increases with advancing age until adult levels are reached at age 15–18 years. Reduction of hemoglobin is usually accompanied by a fall in red cell count and packed cell volume but these may be normal in some patients with subnormal hemoglobin levels (and therefore by definition anemic). Alterations in total circulating plasma volume as well as of total circulating hemoglobin mass determine whether or not anemia is present. Reduction in plasma volume (as in dehydration) may mask anemia; conversely, an increase in plasma volume (as with splenomegaly) may cause anemia even with normal circulating red cell and hemoglobin mass.

After acute major blood loss, anemia is not immediately apparent since the total blood volme is reduced. It may take up to three days for the plasma volume to be replaced and so for the degree of anemia to become apparent. Regeneration of the hemoglobin mass takes substantially longer. The initial clinical features of major blood loss are, therefore, due to reduction in blood volume rather than to anemia.

As per many studies conducted, it is suggested that somewhat lower limits of normal may be used in evaluating the elderly. The lower levels could result from decreased androgen secretion in men or from age related decreases in the stem cell proliferation.

Anemia has to be considered vis-a-vis hematocrit and the red cell mass. Given below are conditions associated with a significant disproportion between hematocrit and the red cell mass.

Relative Increase in Plasma Volume (Hematocrit Disproportionately Low)

- Hydremia of pregnancy
- Overhydration in oliguric renal failure or congestive heart failure
- Chronic diseases and hypoalbuminemia (sometimes)
- Congestive splenomegaly
- Recumbency

Relative Decrease in Plasma Volume (Hematocrit may be High, Normal, or Low, but is High Relative to Red Cell Mass)

- Dehydration, especially saline loss
- Protracted diarrhea (especially in infants), cholera
- Intestinal malfunction (pyloric obstruction), etc.
- Abdominal paracentesis with fluid restriction
- Peritoneal dialysis with hypertonic solutions
- Diabetic acidosis
- Extended fluid deprivation
- Diabetes insipidus with restricted fluid intake
- “Stress erythrocytosis”, spurious polycythemia.

Decrease in Both Plasma Volume and Red Cell Mass (Hematocrit Normal, Red Cell Mass Low)

- Acute blood loss
- Cancer (sometimes)
- Myxedema, Addison's disease, panhypopituitarism.

The effects of changes in posture on red cell concentration may lead to confusion. When normal individuals assume a recumbent position, the hematocrit falls an average 7% (range 4–10%) within 1 hour. When they resume the upright position, the hematocrit increases by a similar amount within 15 minutes. These changes have been attributed to alterations in plasma volume as fluid moves between the circulation and the extravascular spaces in the lower limits as a result of hydrostatic forces. In edematous subjects or in people with low plasma protein or albumin concentration, even greater fluid shifts occur.

PATHOPHYSIOLOGY

Tissue hypoxia reduction in oxygen carrying capacity leads to tissue hypoxia symptoms. Referable to systems with high oxygen requirements, such as skeletal musculature, cardiovascular system and central nervous system are particularly prominent.

Compensatory Physiological Adjustments

- Increased delivery of oxygen to the tissue by the red cell.
- An increase in cardiac output and in the rate of circulation of the blood.
- Maintenance of total blood volume.
- Redistribution of blood flow.

Oxygen delivery to the tissues consists of three components: (1) the hemoglobin in red cells, (2) respiration, and (3) circulation. Each of the three can compensate to some extent for deficiencies in the other components. For example, the rate of circulation accelerates in patients with anemia. Another is that hemoglobin levels increase in hypoxic conditions. The amount of oxygen delivered to the tissues by a given volume of blood is a function of the concentration of hemoglobin. The degree to which it is saturated with oxygen, the affinity of hemoglobin for oxygen, and the tissue oxygen tension. When fully saturated with oxygen, 1 g of hemoglobin binds 1.34 mL of oxygen. At a hemoglobin concentration of 15 g/dL, 100 mL of arterial blood contain about 20 mL of oxygen, whereas the same volume of mixed venous blood contains about 15 mL. The difference results from the extraction of oxygen by the tissues. In anemic patients with a hemoglobin concentration of 7.5 g/dL, only about 10 mL of oxygen can be carried per deciliter of arterial blood. If tissue oxygen consumption is to be maintained in such a patient without adjustments in the circulation, both the percentage saturation of mixed venous blood and the tissue oxygen tension must fall, resulting in an increase in the percentage of arterial oxygen used. It follows, then, that the quantity of blood that must be pumped by the heart to the tissues, per minute, is inversely related to the blood hemoglobin concentration if the other factors do not vary. In fact, however, the hemoglobin–oxygen dissociation curve shifts in chronic anemia. The resulting reduction in the affinity of hemoglobin for oxygen facilitates the delivery of the vital gas to the tissues.

The binding and release of oxygen by hemoglobin are profoundly affected by the variations in the concentration of phosphates, especially 2, 3-diphosphoglyceric acid (2, 3-DPG). The oxygen affinity of hemoglobin falls as the concentration of 2, 3-DPG increases, and rises as it decreases. An increase in red cell level of 2, 3-DPG is found in

chronic anemia. This increase facilitates the delivery of oxygen to the tissues by reducing the affinity of hemoglobin for oxygen at the oxygen tensions found in the capillaries. At alveolar oxygen tensions, the small 2, 3-DPG-induced change in oxygen affinity does not significantely affect the uptake of oxygen by the red cells in the lungs. The net result favors the heart by allowing the tissues to extract oxygen during exercise at a lower cardiac index for an equivalent oxygen consumption and work load than otherwise would be the case. The DPG-induced changes may compensate for up to one-half the expected oxygen deficit in association with anemia.

Cardiovascular Adjustments

The cardiac index generally is increased in patients with anemia, and the arteriovenous oxygen difference is narrowed. The increase in cardiac output is first accomplished by an acceleration in heart rate, rather than by a change in stroke volume. Hence, tachycardia at rest, or with slight exertion, is characteristic of the patient with greater degrees of anemia. Central venous and intracardiac pressures are not altered, although at times the right-atrial pressures may be increased. The total circulating blood volume sometimes, but not always, decreases when the hemoglobin concentration is less than 7 g/dL, the cardiac output is usually increased; when it is less than 5 g/dL, the increase in cardiac index results principally from an increase in stroke volume and, to a lesser extent, in heart rate, especially with exercise. By contrast, in people without anemia, the increase in cardiac output accompanying exercise is caused mainly by tachycardia. In children with severe anemia, the mean cardiac index is approximately the same as in adults with equally severe anemia, however, this state is achieved by greater tachycardia and a lesser increase in stoke volume than in comparably anemic adults. Nevertheless, the capacity of chronically anemic children to tolerate exercise burdens is generally excellent. If congestive heart failure develops, the child usually has some underlying heart disease. Such is not always the case, however, in adults with chronic anemia if the hemoglobin concentration is less than 5 g/dL CHF can develops.

The hyperdynamic circulation in anemia results from diminished vascular resistance. As one consequence, renal blood flow is reduced, leading to salt and water retention. The decreased vascular tone probably results from reduced activity of endothelium-derived relaxing factor (EDRF) or nitric oxide. Hemoglobin normally inhibits EDRF; therefore, the lowered hemoglobin values in anemia lead to increased activity. These alterations may be viewed as a mechanism to control blood pressure in the face of cardiac output.

Cardiac compensation may be marginal in patients with severe anemia, and congestive heart failure may be precipitated by blood transfusion unless precautions are taken. As the cardiovascular system adapts to anemia, the velocity of blood flow tends to increase, thereby shortening the circulation time; this condition may persist even with the development of congestive heart failure. Mild-to-moderate anemia is accompanied by greater coronary flow per unit of left-ventricular work; ultimately, however, when the hemoglobin concentration is reduced below 50% of normal levels, ventricular function is impaired, presumably because the coronary flow has approached its maximum. In contrast to the reflex vasoconstriction that accompanies acute blood loss, the peripheral vascular resistance in chronic anemia falls, a phenomenon that plays a dominant role in the high-output, hyperkinetic circulatory response. Increasing peripheral resistance, as with methoxamine or orthostatic stress, reduces cardiac output. Changes in peripheral resistance are regional, rather than generalized. Muscle flow is increased, whereas skin flow is reduced. Cerebral blood flow is increased. Renal blood flow is diminished. Usually

the systemic and pulmonary artery systolic pressures are unaffected, but the diastolic pressures are lower.

Clinical Features

Patients with anemia usually seek medical advice because of decreased work tolerance, shortness of breath, palpitations, or other signs of cardiorespiratory adjustments to anemia. At times, they feel fine, but their friends or family members may note pallor.

The manifestations of anemia depend on five factors:

1. The reduction in oxygen carrying capacity of the blood.
2. The degree of change in total blood volume
3. The rate at which the previous two factors developed.
4. The capacity of cardiovascular and pulmonary systems to compensate for the anemia and
5. The associated manifestations of the underlying disease that resulted in the development of anemia or any other associated disease.

The hemoglobin concentration is not the sole determinant of the observed symptoms, nor of the urgency with which measures must be taken to correct the anemia. Coexistent cardiovascular or pulmonary disease may aggravate the symptoms associated with a degree of anemia that would be well-tolerated under other circumstances.

In slow developing anemias, symptoms may not appear till hemoglobin falls to 8 or even 6 g/dL. In children, particularly, restriction of capacity for physical exertion may hardly be apparent, despite the presence of severe anemia. The physiologic adjustments that take place chiefly involve the cardiovascular system and changes in the hemoglobin oxygen dissociation curve.

In general, symptoms occur at a higher Hb. level with rapidly developing anemias, e.g. anemia of acute blood loss than in a slowly developing chronic anemia.

Symptoms and Signs

- Tiredness, lassitude, easy fatigability and generalised muscular weakness.
- Pallor of the nailbeds, mucous membranes of the mouth and conjunctivae are more reliable indication of anemia than is pallor of the skin.
- Dyspnea on exertion and palpitations. Angina in the otherwise predisposed individuals.
- Murmurs (hemic murmurs) because of increased velocity of blood flow through valves. These are usually systolic murmurs.
- Systolic bruits over carotid arteries.
- High output state.
- Congestive cardiac failure.
- Slight pedal edema in ambulant patients even without CHF.
- Intermittent claudication.
- ECG changes especially if Hb <6 g% viz. S-T segent depression and flattening or inversion of T waves.
- Faintness, giddiness, headache, tinnitus, spots before the eyes, lack of concentration, and drowsiness; these occur due to CNS involvement.
- Numbness, coldness and sometimes tingling of hands and feet.
- Amenorrhea, menorrhagia in females.
- Loss of libido in the males.
- Anorexia.
- Mild pyrexia 37.2–38.2°C.

- Fundoscopy
- Fundal pallor
- Flame-shaped (blot-shaped) retinal hemorrhages
- Exudates, and
- Impaired wound healing.

The symptoms of acute hemorrhage are those of hypovolemia rather than of anemia. Depending upon amount of blood lost, the patients may experience no symptoms, mild hypotension or progressively severe degrees of shock.

Cardiorespiratory System

In many patients, respiratory and circulatory symptoms noticeable only after exertion or excitment; however, when anemia in sufficiently severe, dyspnea and awareness of vigorous or rapid heart beat may be noted even at rest. When anemia develops rapidly, shortness of breath, tachycardia, dizziness or faintness (particulary upon arising from a sitting or recumbent posture), and extreme fatigue are prominent. In chronic anemia, only moderate dyspnea or palpitation may occur, but in some patients, congestive heart failure, angina pectoris, or intermittent claudication can be the presenting manifestation. A humming or whirring sound in the head, attributed to the rapid blood flow through cranial arteries, may be bothersome to the patient and signal the point at which significant anemia has developed.

With severe prolonged anemia, hyperdynamic cardiac failure may supervene, with salt and water retention, edema, and even ascites. Eventually left ventricular hypertrophy may occur. With correction of the anemia, these abnormalities gradually resolve. The cardiac index falls fastest, usually over a 6-month period. Left ventricular hypertrophy is the last to be corrected, often requiring a year or more.

The rate and depth of respiration often increase even though the mechanical properties of the lungs remain normal. The minute ventilation increases, as does the residual air, but the forced expiratory volume decreases. At rest, arterial oxygen tension is reduced in anemic patients, and as a consequence, the alveolor-blood oxygen tension gradient is greater than normal. During strenuous exercise, a marked depression of both oxygen uptake and carbon dioxide production is observed when anemic individuals are compared with normal subjects exercising at comparable levels.

The oxygen debt incurred during a standard workload and rate of work is greater in an anemic subject than in the same person after recovery from anemia. In the anemic state, it takes longer to restore the heart rate and respiratory minute volume to pre-exercise values than it does after anemia was relieved. The recovery period for oxygen update is prolonged beyond that of carbon dioxide output, reversing the order noted in healthy individuals after exercise. This difference is accentuated by greater degree of anemia and also by greater physical exertion. In the anemic patient, the greater oxygen cost of exercise (that is the volume of oxygen consumed per unit of useful work performed) can be attributed to the greater work required of the respiratory muscles.

The physical signs of chronic anemia may include a bounding pulse and Duroziez sign. Systolic bruits may be heard over the carotid arteries and the cranium, as well as a venous hum, particularly over the internal jugular bulb. Sometimes, by radiation to the chest, the latter may mimic the murmur of a patent ductus arteriosus. These adventitious cardiovascular sounds have been attributed to the greatly increased velocity of blood flow, the reduction in the velocity of the blood, and the turbulence that accompanies these changes.

Heart murmurs are a common cardiac sign associated with anemia. They are systolic in time and best heard in the pulmonic area. Often, they are moderate in intensity, and at times may be rough in quality and raise suspicion of organic valvular heart disease. With severe anemia, rather than simply reflecting increased blood flow and turbulence. The murmurs may be a sign of mitral and tricuspid insufficiency resulting from cardiac dilation. Although these murmurs usually are easily distinguishable from those of organic heart disease, in some cases—particularly those involving sickle cell anemia—it may not be possible to do so. Gallop rhythms of both S3 and S4 type may be detected.

ECG changes can occur in severe anemia and may disappear as anemia is relieved. The most common changes are depression of the S-T segment, and flat or inverted T waves. Changes in the duration of the electrical systole (Q-T interval) and abnormalities of the electrical conduction may be observed. Atrial fibrillation may be associated with severe anemia and revert to normal sinus rhythm when the anemia is relieved.

Skin

Pallor is usually the first sign of anemia, but many other factors besides hemoglobin concentration affect skin color. These factors include the degree of dilatation of the peripheral vessels, the degree and nature of the pigmentation, and the nature and fluid content of the subcutaneous tissues. Certain people routinely have pale-looking skin. The pale appearance of otherwise healthy people residing in tropical and subtropical climates is not the result of a generally lower hemoglobin concentration. Patients with myxedema may present with pallor without anemia. In simple vasovagal syncope, pallor results from cutaneous vasoconstriction. Jaundice, cyanosis, racial skin pigmentation, and dilation of the peripheral blood vessels may all mask the pallor of anemia.

Pallor can be better appreciated in the mucous membranes of the mouth and pharynx, the conjunctivae, the lips, and the nailbeds. In the hands, the skin of the palms first becomes pale, but the creases may retain their usual pink colour until the hemoglobin concentration is less than 7 g/dL. To best appreciate the colour of the hands, they must be normally warm and held at about the level of the heart. A satisfactory judgement cannot be made in the presence of cyanosis or abnormal vasoconstriction.

Character of pallor can assist further. A waxy, dead whiteness of the skin suggests acute blood loss, particularly when accompanied by cold and moist palms. A distinctly sallow color implies chronic anemia. A lemon-yellow coloured pallor suggests advanced pernicious anemia. Definite pallor with mild scleral and cutaneous icterus suggests hemolytic anemia. Marked pallor associated with petechiae or ecchymoses may be seen in leukemias.

Other changes described with anemia are loss of normal skin elasticity and tone, resulting in a dry shriveled appearance. Thinning, loss of luster, and early greying of hair may occur, the last especially in patients with pernicious anemia. The nails may lose their luster, become brittle and break easily (seen especially in chronic iron deficiency anemia, wherein, nails also become concave instead of convex). Chronic leg ulcers may occur, especially in patients with sickle cell anemia and rarely in those with other hemolytic anemias. Glossitis occurs in association with pernicious anemia. When nutritional deficiency is associated with anemia, symmetric dermatitis may develop, fissures may be present at the angles of the mouth, glossitis may occur, and erythematous lesions on the face, neck, hands or elbows may be found.

Neuromuscular System

Headache, vertigo, tinnitus, faintness, scotomata, lack of mental concentration, drowsiness, restlessness, and muscular weakness are common symptoms of severe anemia. Some of these signs may be a manifestation of cerebral hypoxia. In exceptional cases, headache may be as severe as in meningitis. Paresthesias are common in pernicious anemia and may be associated with other symptoms and signs of peripheral neuropathy, and more especially with combined system disease.

Retinopathy

About 20% of anemic patients have flame-shaped hemorrhages, hard exudates, cottonwool spots, or venous tortuousness affecting the retina. The anomalies are not proportionate to the severity of anemia and for unknown reasons are seen more frequently in males. The hemorrhages occur even in the absence of thrombocytopenia. Papilledema related solely to anemia has been described, it clears as the anemia is fully treated.

Gastrointestinal System

Symptoms may occur as a consequence of basic disease (as ulcers) or because of anemia itself, irrespective of the cause. Glossitis and atrophy of papillae of the tongue commonly occur in pernicious anemia and much less often in iron deficiency anemia. Painful, ulcerative, and necrotic lesions in the mouth and pharynx occur in aplastic anemia and in acute leukemia, usually reflecting the neutropenia accompanying these conditions. Dysphagia may occur in chronic iron-deficiency anemia.

Genitourinary System

Slight proteinuria may occur in severly anemia. Microscopic hematuria may be seen in patients suffering from sickle cell anemia.

Other Signs

In severe anemia, the basal metabolic rate may be increased. When anemia is severe, fever of mild degree may occur without other cause. With the exception of acute hemolytic episodes, however, a temperature of 38.3°C (101°F) or more should be taken as a reason to search for infection, lymphoma, and other disorders. When an acute hemolytic episode occurs, jaundice may develop without bile in the urine (acholuric jaundice). If the hemolysis takes place within the circulation, patients may manifest hemoglobinemia, methemalbuminemia, and hemoglobinuria, as well as pain in the abdomen or back and fever. These manifestations are related to the sudden release of hemoglobin and to its destruction and disposal.

History and Physical Examination

History taking in an anemic patient is important and consists of relevant guided questions. The duration of the symptoms and their outset, whether insidious or accute, should be established. Knowing the most recent date at which a hematologic examination indicated anemia was absent is usual. If this information is not forthcoming, a history of acceptance or rejection as a volunteer blood donor or the previous prescription of iron, folic acid, or vitamin B_{12} may help elicit the date of onset of anemia. The family history is important with respect to spells of jaundice, splenectomy, bleeding disorders, and hemoglobin associated abnormalities. The patient's occupation, household customs, and hobbies must be ascertained because certain drugs, solvents and other chemicals may produce hemolytic

anemia or aplastic anemia as well as granulocytopenia and thrombocytopenia. Equally important is place where the patient hails from, e.g. kala-azar is geographically distributed as is schistosomiasis and both can lead to anemia. Many other parasites specific to certain geographic locations can produce anemia and other hematologic disorders.

The patient should be questioned about early graying of the hair, burning sensations of the tongue, sores about the angles of the mouth, and discomfort and brittleness of the fingernails, which are symptoms of the anemias caused by deficiency of specific substances can be treated successfully.

Change in stool habits can provide an important clue to the neoplasms of the colon and rectum underlying the anemia. Patients do not understand the significance of tarry stools and a specific inquiry is essential. The amount of blood lost from hemorrhoids may be overlooked or overestimated. In men occult blood loss is most often from the gastrointestinal tract.

In women, in addition to the preceding, necessary information includes an appraisal of the amount of blood lost during menstruation. Data about number of pads or tampons used and the presence or absence of clots should be ascertained. Menstrual flow should be considered excessive if more than 12 pads are used each period, if clots are passed after the first day or if the period duration exceeds 7 days. The number of pregnancies and abortions and the interval since the most recent of these are also important, for each represents significant iron loss. In a child or adolescent, the rate of growth is noteworthy

The dietary history is very important, but an accurate history is difficult to obtain. You must know what the patient eats or does not eat. Patient may deliberately deceive the physician because of embarassment over eating habits or financial restrictions. Questions regarding the diet must be specific, meal by meal, in the hope of obtaining quantitative information. A record regarding dietary fads and cooking habits may be valuable, especially with reference to folic acid and vitamin C intake. Changes in weight are also very important.

The presence or absence of fever must be known, its presence suggesting infection, lymphoma or other neoplasm or collagen disease. Inquiry regarding cough, pleurisy and hemoptysis is important, because either infection or a lung neoplasm may cause anemia. Pains in the limbs, paresthesias, and difficulty in walking suggest pernicious anemia. Abnormal colour of the urine, suggesting blood or hemoglobin, may signify urinary tract disease or hematologic problems. Bilirubin is not detected in the urine of people with uncomplicated hemolytic anemia, but a darker than normal colour may result from the increased excretion of urobilinogen and its conversion to urobilin.

Bruises, ecchymoses, and petechiae are other important points in the history. Their presence indicates that the disorder producing the anemia may also involve platelets or the liver. Alternatively, the anemia itself may be the consequence of blood loss resulting from a disorder of hemostasis.

In all instances, the presence or absence of symptoms suggestive of an underlying disease such as chronic renal disease, chronic infection, an endocrinopathy, or malignancy must be explored.

The physical examination should include a careful evaluation of the optic fundi and the nervous system. Scleral icterus hints toward hemolytic anemia or ineffective erythropoiesis. Sternal tenderness is a useful and often overlooked sign. In acute leukemia, physical examination often reveals a small, acutely tender area near the middle or lower one-third of the sternum of which the patient may be unaware. A systemic check for palpable lymphadenopathy is necessary, with reference to infection, lymphoma, leukemia, and

metastatic carcinoma. Cardiac murmurs may yield the first evidence of subacute bacterial endocarditis, thereby explaining the presence of anemia. Neither the pelvic nor the rectal examination can be neglected, for tumor or infection in these regions may also cause anemia.

Even when the colour of the urine does not suggest blood, the routine urinalysis should include a test for occult blood. This simple confirmatory procedure should also be performed when hemoglobinuria is suspected or is apparent. A positive reaction, however, may also be due to hematuria or even myoglobinuria. Hematuria may be differentiated from other conditions by finding RBCs on microscopic examinations. Hematuria may be the result of diseases of the kidneys or urinary tracts themselves, or the consequence of thrombocytopenia or of acquired or congenital abnormalities. The sickle cell trait may be accompanied by innocuous hematuria. Hemoglobinuria implies intravascular hemolysis, usually a serious situation. The examiner must not confuse true hemoglobinuria with the release of hemoglobin from RBCs that lyse in the urine after it has been voided.

CLASSIFICATION

There are two main classifications of anemia:

1. The pathogenetic and etiological classifications based on the cause of the anemia, and
2. The morphological classification based on the characteristics of the red cell as determined by blood examination.

Classification of Anemia Based on Pathogenesis and Etiology

Blood Loss

- Acute post-hemorrhagic anemia.
- Chronic post-hemorrhagic anemia.

Impaired Red Cell Formation

Disturbance of bone marrow function due to deficiency of substances essential for erythropoiesis

- Iron deficiency anemia.
- Megaloblastic macrocytic anemias due to deficiency of vitamin B_{12} or folic acid. Anemia associated with malnutrition.
- Anemia associated with scurvy.

Disturbance of bone marrow function not due to deficiency of substances essential for erythropoiesis

- Anemia associated with infection.
- Anemia associated with renal failure.
- Anema associated with liver disease.
- Anemia associated with disseminated malignancy.
- Aplastic anemia.
- Anemia associated with collagen disease, e.g. leukemia, malignant lymphoma, multiple myeloma, myelosclerosis.
- Anemia associated with myxoedema and hypopituitarism.
- Anemia associated with myxoedema and hyperpituitarism.
- Sideroblastic anemias.
- Congenital dyserythropoietic anemias.

Increased Red Cell Destruction (Hemolytic Anemia)

a. Hemolytic anemia due to corpuscular defects (intracorpuscular or intrinsic abnormality).
b. Hemolytic anemias due to abnormal hemolytic mechanism (extracorpuscular or extrinsic abnormality).

Morphological Classification

Based mainly on two features

- The average cell volume (MCV) and
- The average cell hemoglobin concentration (MCHC).

Three Main Types of Anemias are Recognized

- The ***normocytic anemias*** in which MCV is within normal range (76–96 fL). Most normocytic anemias are also normochromic (i.e. MCHC is between 30 and 35 g%) but in some, mild hypochromia may occur.
- The ***hypochromic, microcytic anemias*** in which the MCV is reduced (<76 fL) and MCHC is reduced (<30 g%).
- The ***macrocytic anemias*** in which the MCV is increased (>96 fL). Most macrocytic anemias are normochromic but in some, a mild hypochromia may occur.

Kinetic Classification

The number of erythrocytes present in the circulation at a given time is the result of a dynamic equilibrium between the production and delivery of red cells into the circulation on one hand and their destruction or loss from the circulation on the other. Anemia can be construed to be primarily related to an alteration in production or in destruction/loss, or in both of these factors.

The normal homeostatic mechanisms of the body bring about recovery from anemia by accelerating erythropoiesis (e.g. subsequent to acute blood loss or blood donation), this occurs subsequent to release of erythropoietin and its action on the bone marrow. If the system functions normally, the degree of marrow stimulation is inversely proportional to the degree of anemia. At maximum stimulation, the bone marrow is capable of producing erythrocytes at 6–8 times the normal rate. Based on these principle, a classification of anemia and an approach to the diagnosis have been recommended. An estimate of marrow production using the reticulocyte count is the first step in the process.

Increased red cell production is reflected by an increase in circulating reticulocytes. A reticulocyte production index (RPI) can be easily calculated from hematologic data. The calculation converts the reticulocyte count, expressed as a percentage, to an index reflecting the production rate, expressed as a multiple of normal. Two corrections are incorporated into the calculation, one for the degree of anemia (or the number of nonreticulocytes in the circulation) and another for early release of reticulocytes from the marrow, a process known as shift. Because these shift reticulocytes are released at an earlier stage of their development, they survive as reticulocytes for a longer time in the circulation.

The correction for anemia is made by multiplying the reticulocyte percentage by a ratio of patient's hematocrit (VPRC) to an average normal value. The correction for shift incorporates a value for the reticulocyte maturation time. The reticulocyte production index (RPI), proposed to correct both for shift and for anemia is calculated as per formula given below:

$$\text{RPI} = \frac{\text{Reticulocyte percentage}}{\text{Reticulocyte maturation time (days)}} \times \frac{\text{Patients hematocrit (L/L)}}{0.45}$$

The maturation time of the reticulocyte in the circulation, a measure of shift, is taken to be 1.0 day when VPRC is 0.45 L/L, 1.5 days at 0.35, 2.0 days at 0.25, and 2.5 days at 0.15. For instance, at a VPRC of 0.25 L/L and a reticulocyte count of 20%.

$$RPI = 20/2.0 \times 0.25/0.45 = 5.5$$

Indicating that erythrocyte production is increased to 5.5 times the normal rate.

The average normal RPI is 1, but in the presence of anemia, an increase is to be expected. If the RPI exceeds 3, production is thought to be increased, and the anemia is therefore the result of hemolytic anemia. At RPIs less than 2, a defect in production is assumed. Production defects are of two kinds. Erythropoiesis may be insufficient with an absence, reduction or suboptimal increase in red cell precursors (hypoproliferative). Alternatively, red cell production may be ineffective, a condition characterised by erythroid hyperplasia, but with the production of essentially nonviable red cells. Within the RPI range of 2–3, the kinetic situation is uncertain.

Kinetic Classification of Anemia

Impaired Erythrocyte Production (RPI<2)

Hypoproliferative

- Iron-deficient erythropoieses
- Iron deficiency
- Anemia of chronic disorder

Erythropoietin deficiency

- Renal disease
- Endocrine deficiencies

Hypoplastic anemia

- Aplastic anemia
- Pure red cell aplasia

Infiltration

- Leukemia
- Metastatic carcinoma
- Myelofibrosis

Ineffective

Megaloblastic

- Vitamin B_{12} deficiency
- Folate deficiency
- Other causes

Microcytic

- Thalassemia
- Certain sideroblastic anemias
- Normocytic

Increased Erythrocyte Production (RPI>3)

Hemolytic anemia

- Hereditary
- Acquired
- Treated nutritional anemia.

CHAPTER

4

Basic Principles in Diagnosing and Treating Anemias

INVESTIGATIONS

Before proceeding further one should assess whether or not the patient is anemic. If anemia is confirmed one would like to know the type of anemia that the patient is suffering from and lastly to be able to treat one must clearly understand the cause of anemia.

IS THE PATIENT ANEMIC?

a. Proper history and a thorough examination of the patient are must.

History

History to be sought from every suspected case of anemia.

A complete clinical history should be taken with special reference to the following points:

Age, Sex

Onset

- Rapid or gradual

Blood loss

- Hematemesis
- Malena
- Bleeding hemorrhoids
- Menorrhagia
- Metrorrhagia
- Epistaxis
- Hematuria
- Hemoptysis

Alimentary System

- Appetite
- Weight loss
- Dysphagia
- Abdominal pain
- Diarrhea
- Constipation
- Jaundice
- Soreness of tongue
- History of abdominal operations
- Passage of worms

Reproductive System

- Detailed menstrual history
- Number and interval of pregnancies
- Abortions

Urinary System

- Nocturia

Central Nervous System

- Paraesthesia, difficulty in walking

Bleeding Tendencies

- Easy bruising
- Prolonged bleeding after minor cuts
- Bleeding from many sites

Skeletal System

- Bone pain
- Arthralgia
- Arthritis

Drug Ingestion

Occupational History

Dietary History

Social History

- Alcoholism

Past History

- Previous anemia: Diagnosis, treatment and response to it.

Family History

- Anemia
- Recurrent jaundice

Temperature

- Fever
- Night sweats

a. Estimate hemoglobin—preferably by the cyanmet-hemoglobin method or by the oxyhemoglobin method: Sahli's acid hematin method is obsolete and not a very reliable exercise.
b. Anemia is said to be mild if the Hb is more than 9 g%. Anemia is labeled as moderate if Hb is between 6–9 g%. Any anemia where the Hb is less than 6 g% is severe anemia.

WHAT IS THE TYPE OF ANEMIA?

Careful examination of a well made and stained peripheral blood smear is the single most important test to diagnose the type of anemia. The cell size, shape and degree of hypochromia should be noted. Other features are also good indicators.

CAUSE OF ANEMIA

The cause can be ascertained by correlating the clinical presentation of the patient coupled with results of relevant investigations done in a particular case.

Table 4.1 provides relationship between various clinical pictures with the type/cause of anemia associated with each one of them.

Other investigations which should be done routinely (**Besides Hb and peripheral smear examination)**

a. Preferably about 2–3 mL venous blood should be withdrawn and added to the right anti-coagulant.
b. Do a total and differential while cell count.
c. Perform PCV estimation
   - Enables calculation of MCHC
   - Plasma color gives useful clues as to the etiology of anemia.
d. Reticulocyte count
   - Before and after institution of therapy. A rise in reticulocyte count indicates that a correct diagnosis was made and that the patient is responding well.
   - Raised reticulocyte count occurs
   - With erythroid hyperplasia in the bone marrow: Which may occur naturally as in severe hemolytic anemia or may be a response to therapy given.
   - It may also be increased in disorders causing marrow infiltration.
e. Erythrocyte sedimentation rate
   - Reflects mainly changes in plasma protein pattern. ESR gives a lead regarding an underlying organic cause and is a good prognostic tool.

Table 4.1: System manifestation

	Common to all anemias	*Suggests specific disorders*	*Remarks*
1. Skin and mucous membranes	Pallor Bruising	Bruising Lemon yellow color Follicular petechiae Leg ulcers Fissures of lips Brittle nails, spooning Atrophy of papillae	Inconstant—mucous membranes more reliable. May be seen in any severe anemia. Suggests associated thrombocytopenia When ecchymoses are extensive, they may be cause of anemia (blood loss). Megaloblastic anemia Scurvy Sickle cell disease, chronic hemolytic anemia Iron deficiency Iron deficiency Vitamin B_{12}, folic acid or iron deficiency of tongue, glossitis
2. Cardiovascular	Dyspnea, tachypnea	Dermatitis Thrombotic events tachycardia, palpitations, angina, heart failure, claudication	Nutritional deficiency Sickling disorders, paroxysmal nocturnal hemoglobinuria

Contd.

Table 4.1: System manifestation

	Common to all anemias	*Suggests specific disorders*	*Remarks*
3. Nervous system and eye	Irritability, dizziness, fatigue, headache, tinnitus	Delirium Convulsions Subacute combined Peripheral neuritis Retinal vein tortuosity Retinal hemorrhage Intraocular hemorrhage, retinal vessel arborisation	Vitamin B_{12} deficiency, Thrombotic thrombocytopenic purpura Sickling, lead poisoning Vitamin B_{12} deficiency, degeneration of cord Vitamin B_{12} deficiency, nutritional deficiency, neoplasia Sickle cell disease Vitamin B_{12} or folic acid deficiency, leukemia, may be in any severe anemia or with associated bleeding disorder Sickle cell C disease
4. Gastrointestinal		Anorexia; nausea, flatulence, diarrhea, constipation, jaundice dysphagia splenomegaly	Vitamin B_{12} or folic acid deficiency, may accompany other anemias Hemolytic anemia Iron deficiency anemia Hemolytic anemias, pernicious anemia, iron deficiency, congestive splenogemagly, neoplasia Sickling disorders, spherocytosis
5. Others	Fever	Abdominal pain Bone tenderness Arthritis Gout Limb pain Hypermetabolism	Myeloid metaplasia, leukemia, myeloma Leukemia, collagen vascular disease Myeloproliferative disorders Sickling disorders Leukemia Infection (but may be seen in acute hemolytic anemia, acute leukemia, sickle crisis, pernicious anemia and others).

EXAMINATION OF BLOOD FILM

Method

1. *Mount* Cover the slide using a neutral mounting medium.
2. *Low power field examination look for:*
   - Quality of film
   - Number, distribution and staining of WBCs.
   - For RBCs examination, select an area where they just touch each other without overlapping, i.e. between tail and body of the film.
3. *High power field examination*
   - Assess RBC
     - Size
     - Shape
     - Hemoglobin concentration
4. *Oil immersion examination*
   - Assess atypical cells or note fine details, e.g. inclusion bodies.

Always Note

RBCs (Table 4.2)

Table 4.2: Description and significance of various forms of red blood corpuscles

Type of cell	*Description*	*Physiologic significance*	*Clinical disorders*
Macrocyte	Larger than normal (>7.7 µm in diameter). Well filled with hemoglobin	1. Young RBC (?Skipped generation; early loss of nucleus) 2. DNA synthesis—impaired, megaloblastic maturation	1. Accelerated erythropoiesis. 2. B_{12} or folate deficiency
Thin macrocyte	Diameter increased but MCV normal; often hypochromic (see target cells)	Membrane cholesterol and lecithin increased	Liver disease, posts-plenectomy
Microcyte	Smaller than normal (<6.7 µm)	Differs according to whether or not a. Well-filled with hemoglobin b. Normal in shape	See below
Hypochromic cell	Exaggeration of central pallor (>central 1/3rd); usually also microcytic	Failure of hemoglobin due to: i. Lack of iron ii. Defective globin synthesis iii. Defective porphyrin synthesis	Iron deficiency anemia, anemia of chronic disease (?) Thalassemia, some hemoglobinopathies (C,E) Sideroblastic anemias.
Target cell	Hypochromic, with central pigment; thin cell; surface/volume ratio increased	1. Splenectomy decreases rate and extent of loss of lipids from reticulocytes. 2. Accumulation of both cholesterol and phospholipid on RBC. 3. Congenital	As for hypochromic cells. Also 1. Postsplenectomy 2. In liver disease, especially obstructive jaundice 3. LCAT deficiency Thalassemia
Leptocyte	Thin, hypochromic cell, diameter normal, MCV decreased		
Spherocyte	Spherical, not hypochromic; usually also microcytic; surface/volume ratio decreased, no central pallor	1. RBC membrane abnormality 2. RBCs lose fragment after impact with fibrin strands, walls of diseased vessels and artificial surfaces in circulation	1. Hereditary spherocytosis 2. Acquired immunohaemolytic anemia
Elliptocyte	Elliptical in shape not hypochromic	1. Herediatry abnormality 2. Acquired alteration	1. Herediatry elliptocytosis 2. In various anemias especially megaloblastic.

Contd.

Table 4.2: Description and significance of various forms of red blood corpuscles

Type of cell	*Description*	*Physiologic significance*	*Clinical disorders*
Sickle cell	Sickle-shaped, form assumed under hypoxia (deprivation of oxygen)	Molecular aggregation of HbS	HbS trait or disease also seen in some other hemoglobinopathies Hb C-Harlem, Hb Capetown, etc.
Schistocyte	Helmet or triangular-shaped, fragmented or smaller than normal	RBCs lose fragments after impact with fibrin strands, walls of diseased vessels and artificial surfaces in circulation.	1. Microanglopathic hemolytic anemia 2. Hemolytic anemia due to physical agents 3. Also in uremia, malignant hypertension
"Teardrop" RBC	Shape of drop; usually microcytic, often also hypochromic	Distorted or fragmented RBC	1. Especially in myelosclerosis 2. Less frequently in other forms of anemia, e.g. thalassemia
Spicule cell may be: a. Acanthocyte ('Spur cell')	RBC with tiny projection on surface. Has 5–10 spicules of various lengths irregular in spacing and thickness	Differs according to type: a. Ratio of cholesterol/lecithin of RBC membrane increased when associated with liver disease. Can be converted to normal shape by non-ionic detergents	1. In abetalipoproteinemia 2. Liver disease with hemolytic anemia 3. Postsplenectomy (few) 4. Pyruvate kinase deficiency
b. Echinocyte (Sea-urchin cell, crenated cell, burr cell)	Has 10–30 spicules, evently distributed over surface of the RBC	b. Result of alteration of intra- and extracellular environment. Can be brought about by accumulation of fatty acid or lysolecithin on RBC surface, or both, as a result of changes in plasma or in RBC metabolism	1. Uremia 2. Neonates 3. Pyruvate kinase deficiency 4. Phosphoglycerate kinase deficiency
Pyknocyte	Distorted and contracted RBC, similar to echinocyte		Infantile pyknocytosis
Stomatocyte	Uniconcave, as contrasted with normal biconcave RBC; slit-like instead of circular area of central pallor in RBC	1. Hereditary. Primary defects in membrane structure of function resulting in abnormalities of cation permeability, content and flux 2. Acquired alteration in cation content and flux	1. Hereditary stomatocytosis Several forms 2. Smaller number seen in alcoholic cirrhosis, acute alcoholism, obstructive liver disease, malignancies, etc. and perhaps artifacts.

Size

- Macrocytes, microcytes, normocytes
- Anisocytosis (variation in size)

Shape

Abnormal shape: Oval, pencil, tear, pear, oat and sickle-shaped cells; fragmented cells, target cells, spherocytes, crenated cells, burr cells, acanthocytes, stomatocytes (poikilocytosis).

Hemoglobin

Normochromic, hypochromic.

Immature Forms

Polychromatic, stippled or nucleated red cells.

Inclusion Bodies

Howell–Jolly bodies, Cabot rings, Pappenheimer bodies, malarial parasites.

Arrangement

Autoagglutination, excess rouleaux formation.

WBCs

Number

Normal, increased, decreased.

Abnormal or Immature Forms

Immature forms, hypersegmented macropolycytes, abnormal forms.

Platelets

Number

Normal, increased, decreased.

Form

Abnormalities of size and shape.

TREATMENT

Before embarking on treatment have the accurate diagnosis of the cause of anemia.

Treatment Includes

- Rectification of the disorder causing anemia.
- Administration of specific hematinics.
- Relief from symptoms.

Treatment of Disorder Causing Anemia

1. Arrest of bleeding acute or chronic.
2. Supplement the deficiencies.
3. Relieve the causative systemic disorder. Take care of infection, renal failure, malignancies: Hematological or otherwise, liver disease, autoimmune disorder or endocrine deficiency.
4. Remove the toxic chemical agent or drug.
5. Correct any anatomical gastrointestinal abnormality, if present.

Administration of Hematinics

Folic acid, vitamin B_{12} and iron when given therapeutically are called hematinics. The logic behind giving their extracts is that they contain the important hemopoietic vitamins.

The most common anemia seen in practice is that caused by iron deficiency; iron, therefore, is most commonly needed. Vitamin B_{12} and folic acid deficiencies are on the increase and the anemia caused thereby needs specific vitamins for rectification. In certain disorders non-specific marrow stimulation may be needed and for this testosterone is employed. Erythropoietin (EPO) is now being used to stimulate bone marrow in many situations.

General Considerations

1. Always establish the kind of anemia and its cause before embarking on treatment regimens.
2. Give supplementation of only the specific deficient substance.
3. Give adequate doses of an effective preparation for a sufficient length of time.
4. Do not give shotgun preparations containing iron, vitamin B_{12} and folic acid, etc. For instance, if the patient does improve on shotgun preparations one would not know about the actual substance to which the patient has responded. In the case of vitamin B_{12} and intrinsic factor deficiency, if folic acid is given alone, sub-acute combined degeneration of the cord may be precipitated.

CHAPTER

5

Iron Deficiency Hypochromic Anemia

RELEVANT NORMAL VALUES

Total body iron—3–5 g, more in males.

- Plasma iron—60–70 µg/dL or 10.74–30.43 µmol/L
- Serum ferritin
  - Males 40–430 µg/L
  - Females 14–148 µg/L
  - Children 7–142 µg/L
- Total iron binding capacity (TIBC)—240–450 µg/dL or 42.96–80.55 µmol/L
- Percentage saturation of transferrin—33%
- Red cell protoporphyrin 20–40 µg%
- Bone marrow iron = 1+ to 3+
- (Hb, MCV, MCH, MCHC, etc. have been given in Chapter 3).

HEMOGLOBIN SYNTHESIS IN THE DEVELOPING RED CELL

Iron enters the cell with transferrin and is combined with protoporphyrin, synthesized largely from glycine and succinyl CoA in mitochondria, to form hem. One molecule of hem attaches to one of the globin polypeptide chains and one hemoglobin molecule is made up of four hemoglobin units. Transferrin together with its receptor enters the cell by receptor mediator endocytosis. Iron is released by a fall in a pH and the apotransferrin and receptor are recycled to plasma and membrane, respectively.

IRON METABOLISM

Most body iron is present in hemoglobin in circulating RBCs (Table 5.1). The macrophages of the reticuloendothelial system store iron released from hemoglobin as ferritin and hemodiserin. They also release iron to plasma, where it attaches to transferrin which takes it to tissues with transferrin receptors especially the bone marrow where the iron is incorporated by erythroid cells into hemoglobin. There is a small loss of iron each day in urine, faeces, skin and nails, and in menstruating females as blood. This loss (1–2 mg daily) is replaced by iron absorbed from the diet.

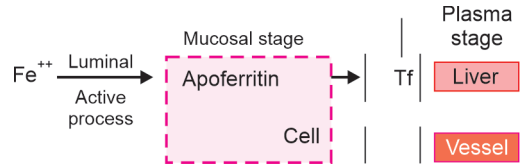
- Both storage iron and percentage saturation of transferrin are:
- Reduced in—iron deficiency anemia.
- Increased in—hemosiderosis and hemochromatosis.

Table 5.1: Distribution of body iron			
Form	*Amount of iron in average adult*		
	Male (g)	*Female (g)*	*Percent of total*
Hemoglobin	2.4	1.7	65
Ferritin and hemosiderin*	1.0	0.3	30
Myoglobin	0.15	0.12	3.5
Hem enzymes			
• Cytochromes	0.02	0.015	0.05
• Catalase			
• Peroxidase			
• Flavoproteins			
Transferrin bound iron	0.004	0.003	0.1

*This is the storage iron available and mobilized, if needed. Rest are part of essential iron—not available for Hb synthesis. The available iron is present equally in bone marrow, liver, spleen, and muscle, etc.

ABSORPTION OF IRON

- It is absorbed in ferrous form (though most iron is available in ferric form)
- It is more in females.
- Ten percent of dietary iron is absorbed normally.



MECHANISM AND CONTROL OF IRON ABSORPTION (Table 5.2; Figs 5.1 and 5.2)

- If apoferritin is completely saturated, absorption is slowed, remaining iron is deposited as ferritin.
- When absorption is slowed, the iron is trapped as ferritin and lost via the body as mucosal cells exfoliate (mucosal block theory).

Table 5.2: Factors influencing iron absorption	
Factors favoring	*Factors reducing*
1. Ferrous form	Ferric form*
2. Inorganic iron	Organic iron
3. Acids: HCl, vitamin C	Alkalis—antacids, pancreatic secretions
4. Solubilizing agents, e.g. sugars, amino acids	Phosphates
5. Iron deficiency	Iron excess
6. Increased erythropoiesis	Decreased
7. Infection	

* Stomach releases iron from food complexes and converts ferric to ferrous form with the help of ascorbic acid and sulfhydryl groups. Erythropoietin has no effect upon iron absorption.

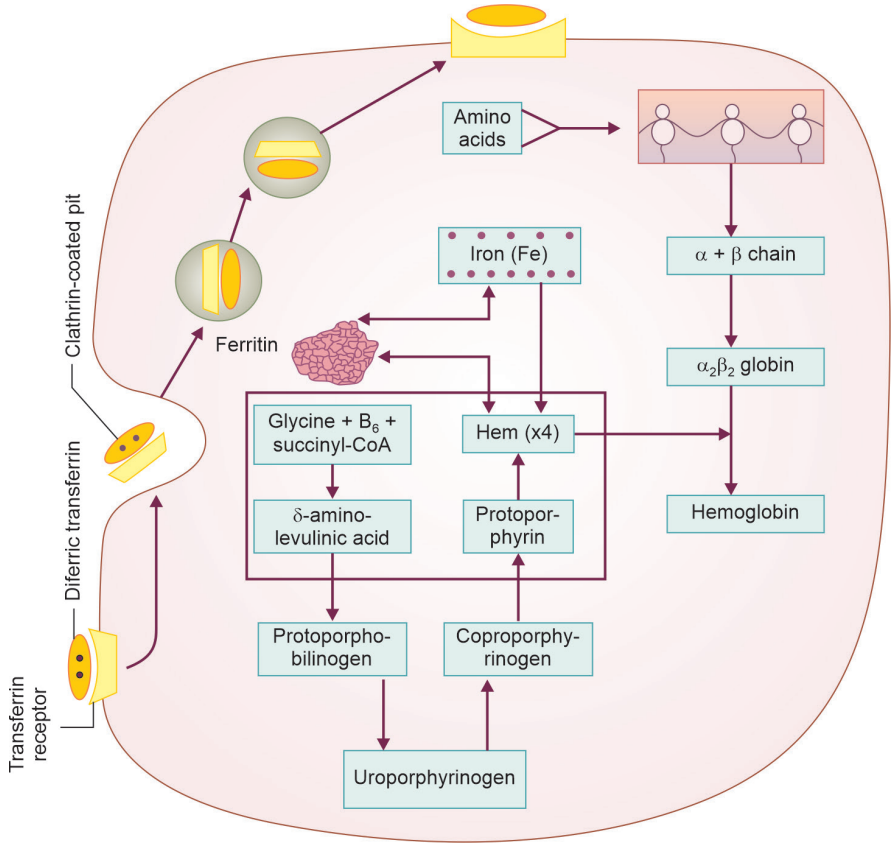


Fig. 5.1: Iron absorption, its control and utilization

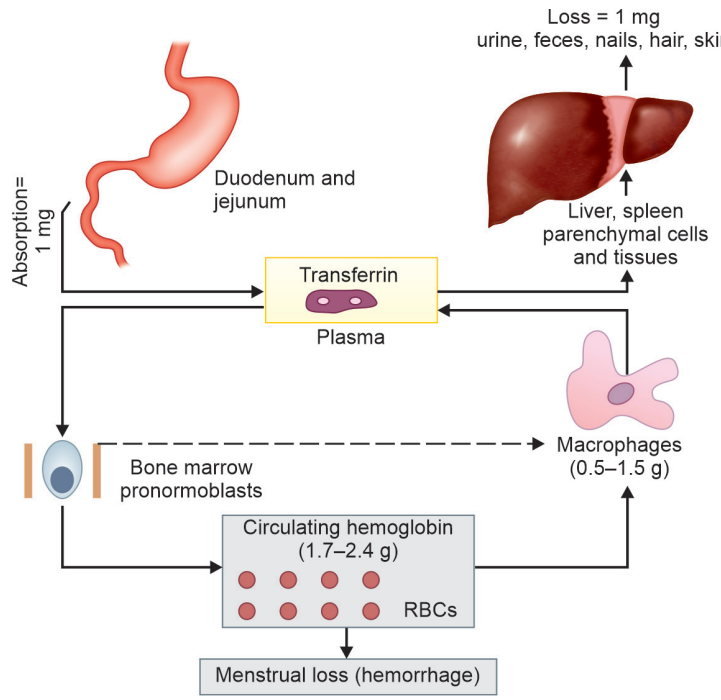


Fig. 5.2: Iron absorption and losses

IRON BALANCE

For requirements of iron in various groups, refer to Table 5.3 and Fig. 5.3.

Table 5.3: Estimated daily requirements, in mg/day

	Urine, sweat, feces	*Menses*	*Pregnancy*	*Growth*	*Total*
Adult male/postmeno-pausal female	0.5–1	0.5–1			
Menstruating female*	0.5–1	0.5–1		1–2	
Pregnant female*	0.5–1		1–2		1.5–2.5
Children (average)*	0.5		0.6		1
Female (12–15 years)*	0.5–1	0.5–1		0.6	1–2.5

*More likely to develop iron deficiency anemia.

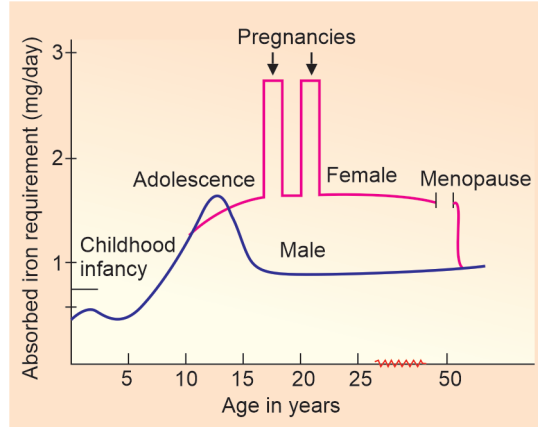


Fig. 5.3: Iron requirements in males and females of various ages

PATHOGENESIS OF IRON DEFICIENCY ANEMIA

Negative iron balance causes decreased iron supply to the bone marrow and hence the anemia.

Various factors responsible for negative iron balance are:

- Blood loss
- Increased requirement during childhood and reproductive life in females
- Impaired absorption
- Inadequate intake.

Iron Deficiency: Stages of Development

A prolonged period of negative iron balance results in iron deficiency. At first, the iron stores in hepatocytes and the macrophages of the liver, spleen, and bone marrow are depleted. Once the stores are gone, plasma iron content diminishes and consequently, the supply of iron to the marrow becomes inadequate for the normal regeneration of hemoglobin. Then, the amount of free erythrocyte protoporphin increases, production of microcytic erythrocytes begins, and the blood hemoglobin level decreases, eventually approaching abnormal levels.

There are three stages:

1. Prelatent
2. Latent
3. Acutal iron deficiency anemia.

Prelatent Iron Deficiency

Prelatent iron deficiency or iron depletion refers to a reduction in iron stores without reduced serum iron levels. This can be detected by assessing iron stores by using biopsy techniques or the measurement of serum ferritin.

Latent Iron Deficiency

Latent iron deficiency is said to exist when iron stores are exhausted but the blood hemoglobin level remains higher than the lower limit of normal. There is reduced transferrin saturation. An increase in the amount of free erythrocyte protoporphyrin is apparent in the middle and later stages of latent deficiency. Other observations include subnormal urinary iron excretion after deferoxamine injection, decreased tissue cytochrome oxidase levels, and increased total iron binding capacity (TIBC). The MCV usually remains within normal limits, however, a few microcytes can be detected on peripheral smear.

Iron Deficiency Anemia

Iron deficiency anemia is said to have developed when hemologin concentration falls below the lower limit of normal. Other iron containing enzymes, such as the cytochromes, also reach abnormal levels during this period. Epithelial manifestations of iron deficiency usually represent a very late phase of iron deprivation.

Cutoff Values of Diagnosing Anemia

The cutoff values of diagnosing anemia as defined by WHO are 14 g/dL for adult men; 12 g/dL for adult women (non-pregnant); and 11 g/dL for adult pregnant women. However, now, values of 11.7 g/dL for nonpregnant women, 13.2 g/dL for adult males are considered correct for diagnosing anemias.

Prevalence

Iron deficiency anemia is more common in women (both pregnant and non-pregnant) and growing children (especially 6 months to 24 months age group). The prevalence of iron deficiency is high in subject groups living in chronic poverty. Probably because of economic factors, iron deficiency tends to run in families. If an iron deficient child is identified, in all probability, the mother and siblings of that child would also be found to be iron deficient.

The iron requirement of an adult male is 5–10 mg/day, while women require 7–20 mg/day (Table 5.3). Poverty, war, unwise agricultural practices, or religious and social tenets aid in increasing the incidence of iron deficiency anemia. Some nations recommend fortification of daily use diets with iron containing salts, etc. The iron salt added should be absorbable but ideally should not affect appearance, taste, or shelf life or the food.

Impaired Absorption

Histamine-fast achlorhydria is common in iron-deficiency subjects, but may be a result of the deficiency as well as a factor in its development. Gastric acid facilitates absorption of ferric iron and food iron, but it has a little effect on hem iron or ferrous iron. Iron deficiency anemia is a common complication of gastric operations, including total gastrectomy, partial

gastrectomy and vagotomy with gastrojejunostomy. Malabsorption associated with celiac disease often leads to iron-deficiency anemia (there is often intestinal blood loss also). Although the rate of iron absorption is usually increased in iron deficient subjects, severe deficiency in infants can be associated with impaired iron absorption. Malabsorption of other nutrients, such as fat and D-xylose, may also occur in severely iron-deficiet infants. Pica is usually a manifestation of iron deficiency and is relieved when the condition is treated. Iron deficiency is a common complication of both clay-eating and starch-eating.

Blood Loss

This is the single most important factor as regards iron deficiency. Identification of source and its apt treatment is equally or may be more important than the anemia itself. 1 mg of blood is considered to contain 0.5 mg of iron. So, a daily blood loss of 3–4 mL/day (1.5–2 mg/day) can result in negative iron balance (Fig. 5.2).

Gastrointestinal Bleeding

It is the most common cause of iron deficiency in men and is second only to menstrual blood loss in women. Occult bleeding is likely to go unnoticed and therefore more likely to cause anemia.

- *Hemorrhoids* are one of the most common ailments, affecting more than 25% of the population. Even if very obvious, the clinician should not end the search for a bleeder at hemorrhoids alone.
- *Upper GI blood loss*—duodenal and gastric ulcers are the most common lesions and the bleeding can be massive or occult. Certain drugs are associated with GI bleeding, of which the non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin are the most important. NSAID administration causes mucosal damage manifested by petechial hemorrhage, erosions, and ulcers. Both gastric and small intestinal tissues are affected. Blood loss can be occult or overt. All NSAIDs have this side effect. Newer drugs are no better than older ones in this respect. These drugs are thought to inhibit epithelial cyclooxygenase with a consequent reduction in prostaglandins with mucosal protective properties. Other drugs associated with gastrointestinal bleeding include stanozolol, anticoagulants, corticosteroids, and ethacrynic acid.

About 15% of patients with hiatus hernia develop iron deficiency, especially, with the paraesophageal variety of lesion and is more common when hernias are large. Colonic diverticulosis and diverticulitis can also cause hemorrhage. Keeping in mind the age and other factors a gastric neoplasm should also be associated with bleeds. Iron deficiency anemia may be the first sign of a neoplasm of the GI tract.

Fair number of ulcerative colitis patients develop iron deficiency anemia. In tropical countries, hookworm infestation (*Necator americanus* or *Ancylostoma duodenale*) is also a significant cause of blood loss. The worms attach to the upper small intestine and suck blood from the host. Schistosomiasis and trichuriasis are other parasitic infections associated with iron deficiency anemia. *Schistosoma mansoni* causes blood loss from the intestine while *Schistosoma hematobium* causes blood loss from the urinary tract.

Menstruation

Most common reason of iron deficiency anemia in women. Average blood flow per cycle is considered to be 35 mL while 80 mL is the upper limit of normal. Women bleeding more than 80 mL and not taking hematinics are likely to be anemic. Intrauterine contraceptive devices (IUCDs) enhance menstrual flow, while contraceptive pills diminish it.

Blood Donation

Regular blood donation is a source of iron loss. Each unit donated, contains 250 mg of iron, which is often enough to exhaust the stores of a normal woman. Three or four such unreplenished donations would exhaust the stores of a normal man. Among donors, the prevalence of deficiency increases with the frequency of donation.

Alveolar Hemorrhage

Hemorrhage into the pulmonary alveoli may cause the blood hemoglobin level to fall 1.5–3 g/dL in 24 hours. Reticulocytosis and hyperbilirubinemia may accompany such episodes, leading to an incorrect diagnosis of hemolytic anemia. Iron thus lost is converted to hemosiderin by pulmonary macrophages, but it cannot be used for hemoglobin synthesis.

Anemia is frequent finding in antibasement membrane antibody disease (Goodpasture syndrome). Eventually most patients develop azotemia.

Other illnesses associated with alveolar hemorrhage include rapidly progressive glomerulonephritis, systemic lupus erythematosus, and certain other collogen vascular diseases and systemic vasculitides. Alveolar hemorrhage is also observed as a toxic reaction to trimellitic anhydride, D-penicillamine, or lymphangiography.

Hemoglobinuria

Urinary iron losses from 2–8 mg/day accompany paroxysmal nocturnal hemoglobinuria. Eventually leading to hypoferremia and hypochromic anemia. Hemoglobinuria in other disorders, such as the erythrocyte fragmentation syndromes associated with prosthetic cardiac valves, also may be complicated by iron deficiency anemia.

Hereditary Hemorrhagic Telangiectasia

Recurrent hemorrhages occur from the nose, GI tract, and other sites. Iron deficiency anemia develops eventually.

Hemostasis Disorders

These disorders rarely cause chronic blood loss leading to hypoferremia. As a rule, the bleeding is acute, and treatment often involves replacement of blood as well as coagulation factors that are leaking.

Chronic Renal Failure Treated with Hemodialysis

Iron deficiency affects 50%, or more of those patients treated with hemodialysis when transfusions are restricted. An important reason is the loss of blood associated with frequent diagnostic tests. Estimated iron losses from these sources is 1.5–2 g/year. GI blood losses from telangiectasias appear to be common. Other factors contributing to negative iron balance include reduced dietary iron intake and iron malabsorption due to aluminium hydroxide used to control hypophosphatemia.

Transferrin saturation is a misleading measure iron metabolism in renal disease, and serum ferritin levels are the best guide to the need of iron supplementation.

Nosocomial Blood Loss

Phlebotomy, especially if repeated, for diagnostic tests can lead to significant blood loss.

Infancy

Etiology of iron deficiency in infancy.

- Decreased total body iron at birth
  - Low birth weight, prematurity
  - Twins
  - Early clamping of umbilical cord
  - Fetomaternal hemorrhage
- Growth
- Inadequate diet
- Blood loss.

Decreased Total Body Iron at Birth

Body iron mass the birth averages 78 mg/kg body weight (range being 65–90 mg/kg), of which 60 mg are in circulating hemoglobin and the rest in stores. Similar concentrations are found throughout fetal development, so a roughly linear relation exists between body iron and body weight. Birth weight of over 4 kg is rarely accompanied by anemia, while birth weight of less than 3 kg is often accompanied by anemia. Iron deficiency is more common in twins as compared to infants born singly. Twins have smaller birth weights. Another factor is occurrence of placental transfusion of one monozygotic twin to another. Early clamping of umbilical cord leads to loss of important blood (as much as 100 mL) present in the placenta. Cord clamping delayed for only 3 minutes can result in 58% increase in red cell volume. If delayed clamping is not practical, as in cesarean sections, a similar effect can be achieved by clamping the cord at the placental end, raising the clamp and allowing gravity to drain the cord. Although the newborn has no immediate need for these erythrocytes, the iron they contain can be used later to meet the demands of growth. Depletion of maternal iron has a little or no effect on the body stores of the newborn. Also, iron supplementation during pregnancy has no effect on the subsequent development of iron deficiency in the infant. Maternal iron levels bear no relation to fetal stores.

Fetomaternal Transfusion

The transplacental passage of fetal erythrocytes into the maternal circulation is a physiologic event. In about half the pregnant women, small amount of foetal blood is found between 21 and 42 weeks of gestation. In the immediate postpartum period about 50% of mothers' peripheral blood shows foetal erythrocytes. In 10% the foetal losses are large and about 1% of fetuses show massive losses of RBCs to the maternal circulation. If fetomaternal loss goes on for a long period, it can cause iron deficiency in the fetus. In a few cases, massive foetomaternal hemorrhage near term may lead to hemorrhagic anemia, shock, or hydrops fetalis in the affected newborns.

Growth

Requirements of adult men, in the absence of disease, are low and vary little. However, in infancy, childhood and adolescence, because of increased needs of rapidly growing tissues, the requirements for iron are relatively great. First year of life places highest demands. The same in premature and low-birth weight infants are still higher. To meet the demands imposed by growth the normal term infant must acquire 135–200 mg of iron in the first year of life. A premature infant may require up to 350 mg during the same period. The relatively slower rates of growth in children from 1 to 12 years of age require a positive iron balance of about 0.2–0.3 mg/day. The sudden growth spurt occurring from 11 to 14 years increases this positive balance demand to 0.5 mg/day in girls and 0.6 mg/day in boys.

Diet in Infancy and Childhood

In an average infant, the iron stores are depleted by 2–6 months of age consequent to demands of growth. During this period, a normal full term infant must absorb 0.4–0.6 mg iron daily from the diet. To achieve this level of absorption, an iron intake of 1 mg/kg/day is recommended for full term infants and 2 mg/kg/day for preterm infants to a maximum of 15 mg/day. *Supplementation, by and large, is a must*. The recommended dietary allowance for children up to 6 years is 15 mg/day; for 6–11 years, 10 mg/day; and during puberty, 18 mg/day. Both, human and cow's milk contain relatively small amounts of iron, however, of what is available, about 50% of iron in human milk is absorbed as compared to 10% from cow's milk. Consequently, infants fed breast milk during the first 6 months of life have greater serum ferritin levels and transferrin saturation than these fed cow's milk; iron deficiency in these children is uncommon. Excessive dependence of infants on unsupplemented cow's milk is an important cause of iron deficiency.

Blood Loss in Infancy

Occult hemorrhage, often without obvious anatomic lesions has been observed in iron-deficient infants. The process is often accompanied by diffuse disease of the bowel with protein-losing enteropathy and impaired absorption of several nutrients. Generalized hypoproteinemia may be observed, along with hypocupremia resulting from ceruloplasmin loss. Considerable evidence shows that the disorder results from hypersensitivity to a heat labile protein in cow's milk. The daily loss of 1–4 mL of blood, along with increased serum albumin turnover occurs while fresh cow's milk is consumed, and these abnormality ceases abruptly with the substitution of heat-treated or soyabean-protein feeding formulas.

An uncommon cause of blood loss, almost unique to infancy is bleeding from Meckel's diverticulum. Diagnosis may be difficult before abdominal exploration.

Pregnancy and Lactation

Each pregnancy results in an average loss to the mother of 680 mg of iron or equivalent to 1300 mL of blood. An additional 450 mg of iron must be available to meet the needs of an expanded blood volume during pregnancy. The latter amount of iron does not represent a net loss after delivery because the iron is returned to stores; it must be available, however, during the pregnancy or else iron deficiency will supervene. On an average the iron requirements over the full term of pregnancy amount to 2.5 mg/day. Most of the requirement occurs during the third trimester. Supplementation is essential. Daily lactational loss is about 0.5–1 mg of iron. The content of human breast milk is probably not affected by the maternal iron stores. Because normal menstruation is usually inhibited while breastfeeding continues, iron requirements in the lactating mother approximate those of the menstruating woman.

IRON DEFICIENCY ANEMIA

ETIOLOGY

Blood Loss

Uterine (menorrhagia, metrorrhagia)
Chronic GIT blood loss, e.g. in

- Esophageal varices
- Hiatus hernia

- Peptic ulcer
- Chronic aspirin ingestion
- Carcinoma of:
  - Stomach
  - Colon
  - Cecum
  - Rectum
- Ulcerative colitis
- Hemorrhoids
- Diverticulosis
- Hookworm infestation (anemia with eosinophilia)

Other Causes of Chronic Blood Loss

- Hematuria
- Repeated epistaxis
- Hemoptysis.

Increased Requirements

- Prematurity (diminished iron stores)
- Growth (Fe deficiency anemia is most common in children 6–24 months of age)
- Females in reproductive age group
  - Menstruation
  - Pregnancy
  - Lactation.

Impaired Absorption

- Achlorhydria (especially in middle-aged females)
  achlorhydria $\rightleftharpoons$ iron deficiency anemia
- Gastrectomy (HCl not available)
- Gastroenterostomy (inflamed anastomosis or intestinal hurry—hence no time for absorption)
- Tropical sprue or celiac disease.

Inadequate Intake

- Improper feeding in infants and young children
- Poverty
- Dietary fads
- Anorexia (nervosa, of pregnancy or malignancies).

Maldistribution

- Atransferrinemia
- Copper deficiency.

CLINICAL FEATURES OF IRON DEFICIENCY ANEMIA

Anemia

- Pearly white sclerae
- Mild splenomegaly in long-standing cases

Epithelial Tissue Changes

- Ozena (nasal mucosal atrophy)
- Koilonychia
- Atrophic glossitis
- Angular stomatitis
- Leukoplakia
- Pruritus vulvae
- Plummer–Vinson syndrome
- Gastric atrophy, gastritis.

CLINICAL PRESENTATION

The clinical manifestation of the underlying disease process with or without those of the iron deficiency state may be observed. About 63% of patients go to a doctor because of symptoms of anemia, 16% visit the physician on account of related disorder causing anemia. In 21% it is an incidental finding.

The onset of iron-deficiency anemia is almost invariably insiduous and the progression of symptoms is gradual. As a result, patients accommodate remarkably well to advancing anemia and may postpone their visit to the doctor for prolonged periods.

Growth

Iron deficiency impairs growth in infancy, and the growth rate is restored on correction of iron deficiency.

Fatigue

Fatigue is a common complaint of anemic patients, and also found in non-anemic patients. With hemoglobin levels of 8–12 g/dL there is usually no correlation between degree of anemia and intensity of the following symptoms: Fatigue, irritability, palpitations, dizziness, breathlessness, and headache. At some point the hemoglobin reaches a level that produces symptoms. Significant number of patients seek medical assistance when hemoglobin levels are between 7 and 8 g/dL.

Some patients with hemoglobin values as low as 4 g/dL, have remarkably a few complaints. When signs do develop, they usually are manifestations of cardiovascular adjustments to anemia. Symptoms may, however, develop in latent iron deficiency too (i.e. iron deficiency without anemia), iron intake significantly diminishes their symptoms.

Neuromuscular Symptoms

Even mild degrees of iron-deficiency anemia impair muscular performance. Total exercise time, maximal work load, heart rate, and serum lactate levels after exercise are all affected adversely in proportion to the degree of anemia. Furthermore, work performance and productivity at tasks requiring sustained or prolonged activity are impaired in deficient subjects which improve when iron is administered.

Even when deficiency is mild, muscle abnormalities are noted. The muscle function is disturbed in iron deficiency.

In anemic children behavioural disturbances are found. These are irritability, short attention spans and lack of interest in their surroundings. Neurologic development in infants and scholastic performance in older children are impaired. With proper therapy all these changes revert back to normal.

The ability to maintain body temperature with exposure to cold is impaired in the iron-deficient patients. About 15–30% of chronic hypochromic anemia complain of neuralgia pains, vasomotor disturbances, or numbness and tingling sensations. Rarely iron deficiency can lead to increased intracranial pressure, papilloedema and the clinical picture of pseudotumor cerebri. It gets corrected after institution of iron therapy. The exact mechanism is not understood but is said that severe anemia and reduced levels of tissue iron enzymes might lead to cerebral anoxia and edema.

Epithelial Changes

Iron deficiency is associated with defective structure or function of epithelial tissue. Nails, tongue and mouth, hypopharynx and stomach are most affected. Iron deficiency can cause generalised pruritis too.

Nails

The fingernails become brittle, fragile or longitudinally ridged. Also seen is thinning, flattening and finally koilonychia, concave or "spoon-shaped" nails. Koilonychia can be induced by caustic chemicals and chronic infections of the nails too.

Tongue and Mouth

Atrophy of the lingual papillae, the most common of the deficiency induced epithelial changes may be accompanied by soreness or burning of the tongue, either spontaneously or stimulated by food or drink, and by varying degrees of redness. Glossitis is found in 15–50% cases of iron deficiency anemia. The filiform papillae over the anterior two-thirds of the tongue are the first to atrophy and may disappear completely. In severe cases, the fungiform papillae also may be affected, leaving the tongue completely smooth and waxy or glistening. One to two weeks of therapy can reverse these changes.

Angular stomatitis is characterised by ulcerations or fissures at the corners of the mouth. It is a less specific sign of iron deficiency than of koilonychia, and it also occurs in riboflavin and pyridoxine deficiencies.

Dysphagia

Paterson and Kelly first described the association of dysphagia, angular stomatitis and lingual abnormalities. Later these findings were confirmed by Plummer and Vinson also. This syndrome can be seen in 5–20% cases of chronic iron deficiency anemia. Patients with sideropenic dysphagia (Paterson-Kelly syndrome or Plummer-Vinson syndrome) note the usually gradual onset of dysphagia; this is described as sharply localised to the area of the neck near the cricoid cartilage. They experience difficulty in swallowing solid foods but have a little problem with the ingestion of liquids. Non-treatment makes it worse to an extent that it interferes with the maintenance of balanced nutrition. The most common anatomic lesion is a "web" of mucosa at the junction between the hypopharynx and the esophagus. These webs which may be multiple, usually extend from the anterior wall into the lumen of the esophagus, but they may encircle the lumen completely, forming a

cufflike structure. In some patients a benign structure, with or without a web, is found, and the opening of the esophagus at the cricoid area may be reduced to a pinhole size or slit. These webs and strictures can be demonstrated radiographically by taking a lateral view of the neck after barium swallow. In mild cases, iron supplementation gives relief, but others may need surgical intervention.

At biopsy, the webs appear to be constructed of normal epithelium with underlying loose connective tissue, within which may be a chronic inflammatory reaction. A few cases may reveal increased mitotic activity in the basal layer. Biopsy of stricture demonstrates chronic nonspecific inflammation and degeneration of striated muscle. A few of these cases may proceed on to malignancy.

Stomach

About 75% cases of iron deficiency anemia reveal gastritis microscopically. It, however, produces a little or no symptoms at all. As atrophy progresses, there is loss of ability to secrete acid, pepsin, and intrinsic factor, in that order. About 33% cases of iron-deficiency anemia develop antibodies to gastric parietal cells.

Immunity and Infection

Lack of iron results in at least two abnormalities in the immune response: Defective cell mediated immunity and impaired bacterial killing by phagocytes. Evidence of defective cell mediated immunity includes as much as 35% reduction in the number of circulating T cells. Both helper and suppressor T cells are affected. These abnormalities can be corrected by administration of iron. Additionally, the production of lymphokines interleukin-1 and interleukin-2 are also impaired in iron deficiency. In neutrophils-phagocytosis, rather than the oxidative burst is considered to become faulty in iron deficiency. Taken together, these abnormalities provide a basis for an expectation that resistance to infection may be impaired in iron deficiency.

Pica

Iron deficient subjects eat ice or other non-food substances. These habits reverse on iron therapy.

Genitourinary System

Menstrual disturbances are common. Whether they are the cause or the result of iron deficiency becomes difficult to ascertain sometimes.

Skeletal System

In children with long-standing iron-deficiency anemia, changes akin to thalassemic skull can be found. The diploic spaces often are widened, and the outer tables are thinned, at times with vertical striations producing a hair-on-end appearance. In addition, abnormalities of the long bones are noted, especially the metacarpals and phalanges, with expansion of the medulla and thinning of the cortices. These changes may result from expansion of the erythroid marrow during bone growth and development.

Spleen

There can be mild splenomegaly (tip just palpable) in about 10 percent of chronic iron deficiency anemia patients.

Peripheral Blood Picture (Fig. 5.4)

- MCV <76 fL
- MCH <27 pg
- MCHC <30 g%
- RBC count normal.

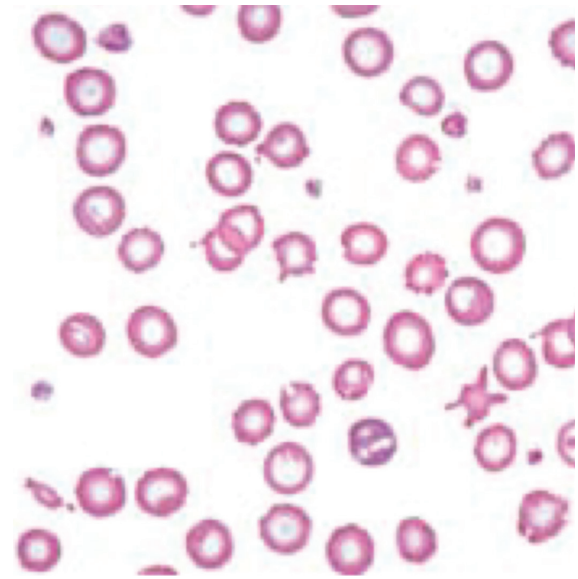


Fig. 5.4: Iron deficiency anemia

Peripheral Smear

- Anisocytosis, microcytic red cells.
- Poikilocytosis, pencil-shaped cells and target cells
- Hypochromia, ring or pessary cells
- A few polychromatophils
- Reticulocyte count is variable.
  - RBC osmotic fragility is slightly decreased.
  - Hematocrit (plasma appears paler) low.
  - Radiochromium-51 Cr studies show reduced red cell lifespan.
  - TLC, DLC and platelet counts are normal.

Bone Marrow

- Micronormoblastic erythroid hyperplasia
- Predominantly intermediate normoblasts seen
- Cytoplasm decreased and shows differential staining
- Cytoplasm matures slowly so that nucleus may be pyknotic while cyptoplasm is still polychromatic
- Bone marrow iron is reduced or absent.

Serum Biochemistry

- Serum iron is reduced (15–60) mg%
- Total iron binding capacity is increased (up to 550 mg%)
- Unsaturated iron binding capacity is also raised.
- Percentage saturation reduced to about 10 percent.
- Red cell protoporphyrin increased (no iron available to form Hb).

Treatment

Iron substitution orally or parenterally.

Response to Treatment

- Reticulocyte count rises (2–16%)
- Hemoglobin rises
- Epithelial changes revert to normal
- Occasionally menorrhagia ceases.

Failure of Response to Oral Iron

May be due to:

- Wrong diagnosis

- Tablets not taken
- Patient non-compliance
- Persistent hemorrhage
- Impaired absorption.

Complicating factors

- Chronic infection
- Chronic renal insufficiency
- Chronic hepatic insufficiency
- Disseminated malignancy.

Management

1. Treatment of iron deficiency and
2. Treatment of the underlying cause

Iron is useful in treating iron deficiency and it has no other legitimate therapeutic use. Iron can be given, intramuscularly or intravenously.

Oral Iron Therapy

Usually ferous sulphate is given orally—as it is effective, well-tolerated and inexpensive. Ferrous fumarate and ferrous gluconate can also be used and they have equivalent side effects. The optimal adult dose is 200 mg of elemental iron given each day. As iron causes gastric irritation, it is advisable to give iron with meals. Absorption is enhanced with citrus fruits/juices, meat, poultry and fish but inhibited by cereals, tea and milk. Iron therapy can be commenced with relatively lower dosages and may be increased gradually—this reduces the severity and incidence of gastrointestinal side effects. For children, the effective dosage is 1.5–2 mg elemental iron per kilogram of body weight 3 times a day. Children usually tolerate elixirs and syrups even on an empty stomach. The 200 mg daily (adult) dose produces a maximal rate of hemoglobin regeneration. It is important to continue oral therapy for 3–6 months after the anemia is relieved, otherwise, a relapse is likely to follow. This continued therapy allows for repletion of iron stores.

Differential Diagnosis of Iron Deficiency Anemia

See Table 5.4.

Table 5.4: Iron deficiency anemia has to be differentiated from other hypochromic anemias

Investigation	*Fe. deficiency*	*Ch. infl/malig*	*Thal.*	*Sideroblastic*
MCV, MCH MCHC	All reduced proportionally to severity of anemia	Low normal or mild reduction	All reduced very low for degree of anemia	Very low in congenital type but MCV often raised in acquired type
Serum iron	Reduced	Reduced	Normal	Raised
TIBC	Raised	Reduced	Normal	Normal
Serum ferritin	Reduced	Normal	Normal	Raised
Bone marrow iron stores	Absent	Present	Present	Present
Normoblast iron	Absent	Absent	Present	Ring form
Hb electrophoresis	Normal	Normal	HbA_2 raised	Normal

Side Effects

Some patients on being given oral iron complain of gastrointestinal symptoms, including heartburn, nausea, abdominal cramps and diarrhoea. About 100 mg of elemental iron per day usually does not produce significant side effects. Other preparations available are a polysaccharide-iron complex and carbonyl iron. Carbonyl iron offers the benefit of lesser side effects. If given in sufficient quantities, both ascorbic acid and succinic acid increase absorption of ferrous iron. Enteric coated or sustained release medications may reduce side effects but they are also less likely to be absorbed adequately.

Failure to Respond to Oral Iron

Reasons can be (1) incorrect diagnosis. (2) complicating illness (3) failure of patient to take prescribed medicine (4) inadequate prescription (dose or form) (5) continuing iron loss in excess of intake and (6) malabsorption of iron.

Parenteral Iron Therapy

Parenteral iron therapy is effective but more dangerous and considerably more expensive. Parenteral iron therapy is indicated when the patient (a) is unable to tolerate iron compounds given orally (b) repeatedly fails to heed instructions or is incapable of accepting or following them (c) loses iron at a rate too rapid to be compensated for by oral therapy; as may occur in hereditary hemorrhagic telangiectasia, (d) has a disorder of gastrointestinal tract, such as ulcerative colitis in which symptoms may be aggravated by oral iron therapy (e) is unable to absorb iron given orally. (f) is unable to maintain iron balance on treatment with hemodialysis or (g) is donating huge amounts of blood for autotransfusion programs.

Iron Dextran Complex

Iron dextran complex, which contains 50 mg of iron per mL of solution, is the preparation of choice. The total dose is calculated from the amount of iron needed to restore the hemoglobin deficit plus an additional amount to replenish the stores.

$$= \frac{\text{Iron to be injected (mg)}}{(15 \text{ patients Hb in g/dL})} \times \text{body weight in kg} \times 3$$

Iron-dextran can be given intramuscularly or intravenously. The intramuscular injections are best made deep into the upper outer quadrant of the buttock, and the skin should be laterally displaced before injection (z-track technique) to prevent staining of the skin. A dose of 0.5 mL should be given to test for hypersensitivity. After a day, or at least one hour, the full dose may be given. The maximal recommended daily dose is 2 mL (or 100 mg). About 75% of injected dose is absorbed from injection site and 25% may remain there itself and be essentially unavailable.

Iron-dextran complex can be given intravenously too. After testing for hypersensitivity, 2 mL of undiluted iron-dextran complex may be given daily at the rate of 1 mL/minute. Or else the total iron dose can be given in a single infusion (TDI or total dose infusion). 5 mL of iron dextran complex is diluted with 100 mL of normal saline. The whole dose can be given as a single infusion or may be broken into three parts to be given intravenously on three consecutive days. The initial flow should be 20 drops/min for 5 minutes, if no side effects are observed, the rate may be increased to 40–60 drops/minute.

Side Effects

These can be local and/or systemic. Local reactions to intramuscular injections include pain at the injection site and discolouration of the skin. Regional lymph nodes may become tender for several weeks.

When intravenous injections of more than 100 mg/minute are infused, there can be pain in the vein injected, flushing, and a metallic taste. Such reactions are brief in duration and often are relieved immediately by slowing the infusion rate.

Systemic reactions to both intramuscular and intravenous injections are either immediate or delayed. Immediate side effects include hypotension, headache, malaise, urticaria, nausea and anaphylactoid reactions. Delayed reactions include lymphadenopathy, myalgia, arthralgia, and fever. Most of the reactions are mild and transient but anaphylactoid reactions can be life-threatening.

Response to Treatment

Much before the anemia, the other related symptoms improve. Patients may show rapid subjective improvement, with disappearance or marked decrease in the sense of fatigue, lassitude, and other nonspecific symptoms. Pica gets relieved within a week or two.

The earliest hematologic response to treatment is reticulocytosis. The increase generally is slower to appear and is not so marked as may be observed after vitamin B_{12} therapy for pernicious anemia. Reticulocytosis reaches 5–10% on the fifth to tenth day after institution of therapy and thereafter gradually returns to normal. Rate of hemoglobin increase is higher in severe anemias and about 2 months elapse before it returns to normal. The hemoglobin value should increase to a point half way between starting level and normal in an average of 18 days; and at 3 weeks, the hemoglobin value should have risen 59 ± 17% toward normal. Hemoglobin estimation is the most accurate representation of the degree of anemia in iron deficiency. During the response to therapy, the red cell count may increase temporarily to values above normal, but the hemoglobin value lags behind. The red cell indices may remain abnormal for sometime after the normal hemoglobin level is restored. As recovery takes place, the normocytic cell population gradually replaces the microcytic cell population. Of the epithelial lesions, the first to respond are, those affecting the tongue and nails. After 1–2 weeks, small regenerating filiform papillae are observed, and after six months, the tongue reverts back to normalcy; however, in severe anemias, some atrophy may persist. Koilonychia usually disappears in 3–6 months, the concavity moving towards the end of the nails as the nail grows. Gastritis and the associated defects in gastric secretion often do not respond to therapy, especially in aged individuals. In under 30 years individuals, gastric acid secretion and normal epithelial architecture may be restored.

Dysphagia may be relieved by iron therapy if the associated postcricoid webs are small or medium sized. The webs themselves are not altered, however, and relapse of the anemia is associated with recurring dysphagia. With more severe lesions, dilatation of the esophagus is needed to provide relief.

Relapse occurs in about 33 percent of the patients who respond to iron therapy, in part because of failure to complete the full course of treatment and in part because of recurrent disease.

Preventive Therapy

High-risk individuals need prophylactic measures. Among these persons there are infants, pregnant women, adolescents, regular blood donors, women with menorrhagia, and patients receiving continuous, high dose aspirin therapy.

PLUMMER–VINSON SYNDROME (PATERSON–KELLY SYNDROME)

- Characterized by chronic iron deficiency anemia and dysphagia often with glossitis.
- Usually occurs in middle-aged or elderly women.
- Anemia tends to be severe and spleen tip may be palpable.
- Dysphagia may be due to spasm of the oesophageal entrance or due to fine web/band formation (made up of desquamating epithelial cells). Solid foodstuff cannot be swallowed and only liquid or soft foods can be taken.
- Dysphagia gets worsened if the patient is tired.
- Iron alone may improve dysphagia or one may need to pass boggie through the site of obstruction.
- The mucosal change may ultimately develop into postcricoid carcinoma.

INVESTIGATIONS OF A PATIENT WITH IRON DEFICIENCY ANEMIA

HISTORY

Menstruating Females

Menstrual history
- Menorrhagia
- Metrorrhagia

Pregnancies
- Number and interval
- Abortions

Male and Post-menopausal Women

Diet

Alimentary blood loss
- Symptoms suggestive of GI disorder
  - Weight loss
  - Anorexia
  - Dyspepsia
  - Abdominal pain
  - Diarrhea
  - Constipation
  - Change in bowel habits
  - Dysphagia
  - Acid regurgitation
  - Worm infestation (hookworms)
- Hemorrhoids
- Hematemesis
- Melena
- Gastrointestinal surgery
- Epistaxis
- Hematuria
- Hemoptysis
- Chronic aspirin ingestion

Infants and Children

Detailed dietary history, especially of supplemental feeding, prematurity, multiple births, iron deficiency in the mother.

- Gastrointestinal disturbance
- Blood loss
- Infection, worm infestations.

PHYSICAL EXAMINATION

- Abdomen
  - Abdominal mass
  - Tenderness
  - Hepatomegaly
  - Splenomegaly
- Rectal examination and proctoscopy
- Pelvic examination
- Telangiectasia of face and mouth.

SPECIAL INVESTIGATIONS

Disorders for which particular investigations are required, are given within parenthesis.

Investigations Needed Commonly

- Stool routine and microscopic examination (ova of worms). Stool test for occult blood to be repeated, if necessary.
- Barium meal (peptic ulcer, hiatus hernia, carcinoma of stomach)
- Barium swallow (esophageal varices)
- Barium enema (carcinoma colon, cecum, ulcerative colitis)
- Sigmoidoscopy (carcinoma rectum, ulcerative colitis)
- Microscopic examination of urine (hematuria)
- Chest X-ray (disorders causing hemoptysis).

Investigations Needed Occasionally

- Cytoscopy and/or pyelography (disorders causing hematuria)
- Liver function tests (cirrhosis of liver)
- Fat balance test, jejunal biopsy (steatorrhea)
- Laparotomy (persistent unexplained gastrointestinal bleeding).

IRON OVERLOAD

HEMOSIDEROSIS

Hemosiderosis is the accumulation of iron to supranormal levels, producing gross hemosiderin deposition in macrophages of the spleen, liver, bone marrow, and other tissues. In hemosiderosis iron deposition is predominantly in reticuloendothelial cells, whereas the iron overload in hemochromatosis leads to deposition in parenchymal cells. The most important causes of hemosiderosis are hemolytic anemias, multiple blood transfusion, and disorders of erythropoiesis (megaloblastic anemia, sideroblastic anemia, and thalassemias). Chronic exposures to high dietary iron and increased iron absorption caused by chronic alcohol abuse can also generate systemic iron overload. In general, the reticuloendothelial iron deposition in hemosiderosis is not usually accompanied by organ damage but may lead to hemochromatosis, in which such damage can occur. An increase in iron stores is usually associated with high percentage saturation of transferrin, except in situations of

chronic disease. Desferrioxamine-induced urinary iron excretion is generally greater than 4 mg/day (normal being <2 mg/day). Desferrioxamine may also be used in the therapeutic management of the iron overload states.

HEMOCHROMATOSIS

Hemochromatosis is a more severe form of iron accumulation that has progressed over a long period of time to involve widespread parenchymal iron deposition and organ injury. It presents classically as a triad of skin pigmentation, diabetes mellitus and hepatomegaly (often cirrhosis), with or without heart disease and hypogonadism. This has led to the classic terms "bronze diabetes" and pigment cirrhosis. The bronze skin colour is due primarily to an effect on the melanin synthesis and is not due to frank iron deposition in the skin. Morbidity is most commonly related to cirrhosis and diabetes when the disease is advanced and there is an elevated incidence of hepatocellular carcinoma. Heart failure and arrhythmias may also develop. Hematologic consequences of hemochromatosis are a few. Serum iron may be elevated tenfold or more, with transferrin saturation approximating 100%, and total body stores may be 20–100 g. Desferrioxamine-induced urinary excretion ranges from 5 mg/24 hr to more than 20 mg/24 hr. The standard therapy is frequent and periodic phlebotomy in an attempt to induce iron depletion except when iron overload is secondary to transfusions for anemia conditions. In the latter case, chelation therapy with desferrioxamine is used to reduce iron deposition.

SIDEROBLASTIC ANEMIAS

Sideroblastic anemias are a heterogenous group of disorders characterized by the presence of amorphous iron deposits in the form of ferric phosphate and ferric hydroxide in erythroblast mitochondria. The iron-laden mitochondria often assume a perinuclear distribution, particularly in the later stages of erythroblast maturation, and account for the so-called ringed sideroblast, a nucleated red cell in which large prussian blue-positive granules form a full or partial ring around the nucleus.

The basis for the mitochondrial iron accumulation in the various sideroblastic anemias is considered to be insufficient generation of hem as a result of decreased production of protoporphyrin or impaired insertion of iron into protoporphyrin in the erythroid cell. Globin synthesis is also impaired, but this effect is secondary as it can be corrected by the addition of hem *in vitro.* Iron delivery to the erythroid cell is not down regulated in the fact of the diminished hem synthesis, and iron continues to be transported normally to mitochondria, where it accumulates.

Kinetically, the sideroblastic anemias are characterized by ineffective erythropoiesis, like other erythroid disorders with defective cytoplasmic or nuclear maturation. Erythroid hyperplasia of the bone marrow is accompanied by a normal or only slightly increased reticulocyte count. The plasma iron turnover rate is increased but iron incorporation into circulating red cells is reduced. Red cell survival tends to be normal or slightly reduced. Mild hyperbilirubinemia may be noted, as well as an increase in urobilinogen excretion as a result of raised erythropoietic component of the "early label" bilirubin peak. Thus it can be inferred that a substantial proportion of the developing ringed sideroblasts are non-viable and their destruction within the marrow accounts for the kinetic abnormalities.

The progeny of the ringed sideroblasts are hypochromic and microcytic erythrocytes, a finding that provides morphologic evidence of impaired hemoglobin formation as well as the initial clue to the diagnosis. The degree of microcytosis varies considerably from one form of sideroblastic anemia to another. Often, dimorphism is pronounced, with a

hypochromic/microcytic population of cells co-existing side by side with a normal or even a macrocytic one, the siderotic mitochondria of the developing cell may be retained in some circulating erythrocytes (Pappenheimer bodies), and are regularly found with concomitant hypofunction or absence of the spleen; these cells are nearly pathognomonic siderocytes in the usually Wright's stained peripheral smears. A constant feature of those sideroblastic anemias that are not reversible is an increase in total body iron. The serum iron concentration is increased, often to the point of complete saturation of transferrin, and the level of serum ferritin roughly reflects the degree of iron overload. The ineffective erythropoiesis mediates, in an unknown manner, increased intestinal absorption of iron. The consequent iron overload state is called erythropoietic hemochromatosis and its clinical and pathologic features and course are indistiguishable from those of hereditary hemochromatosis.

Iron containing nucleated red cells are called sideroblasts and red cells are called siderocytes.

Classification of the Sideroblastic Anemia

Hereditary Sideroblastic Anemias

- X-linked
- Autosomal dominant
- Autosomal recessive
- Congenital
  - Sporadic, inheritance undetermined
  - Associated with mitochondrial cytopathy (Pearson syndrome)
  - DIDMOAD syndrome

Acquired Sideroblastic Anemia

- Idiopathic
- Associated with myelodysplasia, hematologic malignancies, myeloproliferative disorders and chemotherapy

Reversible-associated with

- Alcoholism
- Certain drugs (isoniazid, chloramphenicol)
- Copper deficiency (nutritional, zinc toxicity, copper chelation)
- Hypothermia.

Types of Sideroblasts

Type I

- Present in normal bone marrows
- Have scattered small granules (difficult to see)
- Granules not localised to perinuclear zone.

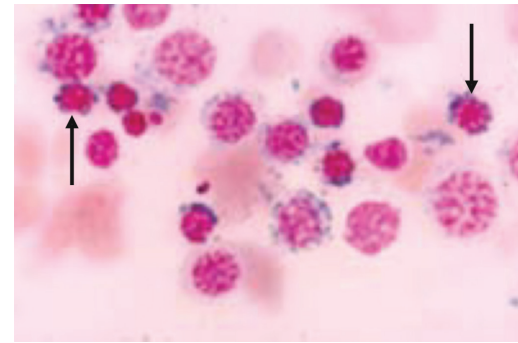


Fig. 5.5: Pathological sideroblasts

Type II

- Abnormal, seen when percentage saturation of transferrin is increased.
- Granules are larger, numerous and diffusely scattered.
- Seen in hemolytic anemia, megaloblastic anemia, hemosiderosis and hemochromatosis (Fig. 5.5).

Type III

- Not related to percentage saturation of transferrin
- Granules still larger and most numerous
- Distribution of granules scattered
- Ring around nucleus
- In these there is disturbed Hb synthesis, e.g. in
  - Primary/secondary sideroblastic anemia
  - Thalassemia

Hereditary Sideroblastic Anemia

Pseudothalassemia

- Sex linked, males are affected, females are carriers
- Marked anemia, onset in childhood/young adults
- Normocytic, hypochromic anemia; both MCV and MCHC are reduced
- TIBC is normal or completely saturated
- Bone marrow-increased number of ring sideroblasts
  - Micronormoblastic reaction
  - Iron deposition increased in bone marrow and other tissues.

Acquired Primary Idiopathic Sideroblastic Anemia

- Affects both sexes, middle-aged/elderly, insidious onset.
- Generalized skin duskiness.
- Moderate anemia, dimorphic—both normochromic; normocytic and microcytic, hypochromic red cells seen. RBC lifespan is decreased.
- A few target cells, polychromatophils, poikilocytes and schistocytes seen.
- Reticulocyte count, MCHC, MCH, TLC, platelet counts are normal or decreased.
- Serum bilirubin and percentage transferrin saturation are normal or increased.
- Bone marrow
  - Erythroid hyperplasia.
  - Marked increase in sideroblasts, especially ring forms.
  - Reaction normoblastic, ± megaloblastic
  - Erythropoieses show shift to the left.
  - There is ineffective erythropoiesis.

Secondary Sideroblastic Anemia

Like acquired disorder.

Treatment

No Iron Therapy

- Pyridoxine, folic acid, crude liver extract, transfusion
- In patients with iron overload
- Desoxyferramine
- Venesection.

RADIOACTIVE ^{59}Fe STUDIES

1. ^{59}Fe red cell utilisation—for diagnosing effective function.
2. Surface counting studies—for myeloid metaplasia.
3. Plasma iron turnover—for ineffective erythropoiesis.

BONE MARROW IRON

Bone marrow iron can be appreciated directly in unstained preparations of aspirations as the golden refractile granules of hemosiderin. More reproducible assessments are obtained with Prussian blue staining, which renders the hemosiderin blue. Histologic grading (0 to 6+) correlates reasonably well with iron content of the sample.

1+ to 3+ are considered normal.

0 is considered iron deficient.

4+ to 6+ are present in iron overload states

2+ to 5+ are seen in anemia of chronic disease (conversely, anemia of Fe deficiency shows grade 0)

2+ to 4+ in thalassemia minor and in many hemoglobinopathies.

5+ to 6+ in thalassemia major, sideroblastic anemias, and hemochromatosis.

Criteria for Grading Iron Stains in Bone Marrow Aspirated (Table 5.5)

Table 5.5: Bone marrow iron staining-criteria

Grade	*Criteria*
0	No iron granules observed
1+	Small granules in reticulum cells seen only with oil immersion lens
2+	A few small granules visible with low power lens
3+	Numerous small granules in all marrow particles
4+	Large granules in small clumps
5+	Dense, large clumps of granules obscuring marrow detail
6+	Very large deposits, obscuring marrow cells

CHAPTER

6

Megaloblastic, Macrocytic Anemias

RELEVANT NORMAL VALUES

MCV	= 76–96 fL
Unsaturated B_{12} binding capacity	= 500–1200 ng/L
Saturation of Transcobalamin I	= 70–100%
Serum vitamin B_{12}	= 160–925 ng/L
(microbiological assay)	
(radioisotope assay)	= 200–300 ng/L
Schilling test, normal excretion >10% of oral dose	
Serum folate (microbiological assay)	= 6–12 µg/L
Red cell folate	= 160–640 µg/L
Neutrophil nuclear segmentation	= 2–5 lobes
Serum LDH	= 71–207 IU/L
Serum bilirubin	= 0.2–1 mg%

In megaloblastic anemias there is cytological and functional abnormality both in peripheral blood and bone marrow.

VITAMIN B_{12} KINETICS

- Acid and pepsin release B_{12} from food complexes
- Its absorption may be passive or active through intrinsic factor (IF).
- Parietal cells of stomach secrete intrinsic factor
- Vitamin B_{12} and IF bind in 1:1 ratio
- In the presence of calcium, vitamin B_{12} attaches to the brush border (distal ileum) in pH >6, it is then taken into the intestinal mucosa.
- At supraphysiologic dose, equal absorption occurs through jejunum.

Table 6.1 gives the nutritional factors, daily requirements, absorption sites as related to vitamin B_{12} and folic acid.

Table 6.1: Nutritional factors, vitamin B_{12} and folate

	Vitamin B_{12}	*Folate*
Adult daily need	2 µg	100 µg
Normal dietary intake	7–30 µg	600–1000 µg

Contd...

Table 6.1: Nutritional factors, vitamin B_{12} and folate		
	Vitamin B_{12}	***Folate***
Main foods	Animal produce only	Meat mainly liver, yeast and green vegetables
Cooking	Little effect	Easily destroyed
Body stores	2–3 mg (enough for 2–4 years)	10–12 mg (enough for 4 months)
Absorption site	Distal ileum	Duodenum and jejunum
• Mechanism	Intrinsic factor	Conveersion to methyltetrahydrofolate
• Limit	2–3 mg daily	50–80% of dietary content
Major intracellular	Methyl and adenosly cobalamin	Reduced polyglutamate derivatives
Usual therapeutic from	Hydroxocobalamin	Folic acid

Transport

TC I (Transcobalamin I)

- It is an α_1 globulin and it carries 70–90% circulating Vitamin B_{12}
- Normal saturation of TC-I is 70–100%

TC II

- It is a α-globulin prepared in liver
- It is needed for transport of vitamin B_{12} from one organ to another and from in and out of the cell.
- It is largely saturated.

TC III

- It has been recognized recently.
- UBBC
- Unsaturated B_{12} binding capacity (UBBC) reflects availability of TC-I and TC-II in serum, normal UBBC = 500–1200 ng/L.
- UBBC rises in chronic myeloid leukemia, acute promyelocytic leukemia and other myeloproliferative disorders.

Tissue Stores

Liver, kidney, heart and brain.

Excretion

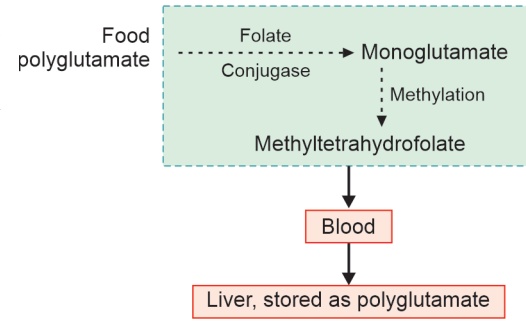
Through bile and hence undergoes enterohepatic recirculation.

Function

- Isomerization of methylmalonyl CoA to succinyl CoA
- Methylation of homocysteine to methionine
- In vitamin B_{12} deficiency urinary excretion of methyl malonic acid increases
- The homocysteine methionine reaction is closely linked with the metabolism of folic acid, hence vitamin B_{12} deficiency would cause impaired conversion of methyltetrahydrofolate to tetrahydrofolate.

FOLATE KINETICS

- Chemically it is polyglutamic acid (PGA)
- Folate circulates as methyltetrahydrofolate monoglutamate
- It is stored in liver but is also present in RBCs.



Function

- For transfer of one carbon units
- For conversion of formiminoglutamic acid to glutamic acid
- Methylation of homocysteine to methionine
- For conversion of deoxyuridylate monophosphate to thymidilate monophosphate.

(Increased excretion of formiminoglutamic acid in deficiency of folic acid is the basis of FIGLU test).

VITAMIN B_{12} DEFICIENCY

Pernicious anemia is seen worldwide and occurs due to deficiency of Castle's "intrinsic factor" in gastric secretions. Lack of this factor (binding protein) impairs absorption of vitamin B_{12} leading to its deficiency and eventually anemia.

Pernicious anemia is seen commonly in persons in late adult life. It rarely affects individuals younger than 30 years and increases in frequency as age advances. A rare congenital form is also identified (appears before the age of 2 years) and there is total lack of intrinsic factor molecule, but the structure and function of gastric mucosa are otherwise normal, and no antibodies against gastric parietal cells are recognised another form known as juvenile pernicious anemia is also known where the disease manifests itself in the second decade of life. Gastric atrophy is noted and it is identical to the adult form. These conditions must be differentiated from megaloblastic anemia occurring in neonates as the result of congenital deficiency of transcobalamin II or other inborn errors of metabolism.

ETIOLOGY AND PATHOGENESIS

The fundamental abnormality is severe atrophic gastritis, leading to extreme deficiency of all gastric secretions including intrinsic factor, essential for the absorption of vitamin B_{12}. The gastric lesion probably develops as a result of an autoimmune attack on gastric cells. The basis of this may be genetic or gastritis secondary to other causes.

Genetic Factors

Congenital form of pernicious anemia is inherited, probably as an autosomal recessive trait. The adult form of disease is not found to be familial. Genetic factors, however, do play a role in the etiology of adult pernicious anemia, the same is not discretely understood. A weak association has been made between the histocompatibility locus antigen HLA-B7 in some cases. The association of HLA-D antigens, especially Dw_2, Dw_5 and DR_2 is stronger and statistically significant.

Gastritis

The indicence and severity of gastritis rises with advancing age. As the disease progresses, hydrochloric acid, pepsin, and intrinsic factor are lost, in that order. Patients with these

forms are usually asymptomatic. Patients with severe atrophic gastritis or with gastric atrophy may develop vitamin B_{12} malabsorption.

Causes of simple gastritis include:

1. Chemical, thermal and mechanical injury to the mucosa or caused by *Helicobacter pylori.*
2. Nutritional deficiency-iron, folate, and ascorbate
3. Endocrinologic insufficiency—thyroid, adrenal, and pancreatic
4. Genetic abnormalities, and
5. Autoimmune disorders.

Immunologic Mechanism

About 85% cases of pernicious anemia have parietal cell antibody, 50% cases of gastritis also have this antibody and about 10–15% of normal subjects may also possess this antibody (many of these may, have asymptomatic gastritis). Two types of parietal cell antibodies exist: 1. Directed against a cytoplasmic antigen and 2. React with cell surfaces; the latter is cytotoxic to gastric mucosal cells in the presence of complement. Intrinsic factor antibodies occur in the serum of approximately 55% of patients with pernicious anemia (these antibodies are more specific to pernicious anemia). The most common type includes the so-called blocking antibodies, which prevent vitamin B_{12} from complexing with intrinsic factor and are therefore presumed to combine with the intrinsic factor molecule at or near the B_{12} binding site. Binding antibodies have also been detected. All of these antibodies belong to the IgG class. Intrinsic factor antibodies also can be detected in the gastric juice of 75% of subjects with pernicious anemia. They may be found even in the absence of a corresponding serum antibody. They may be either of IgG or the secretory IgA type (i.e. produced by gastric mucosal plasma cells). Thyroid antibodies frequently are found in patients with pernicious anemia and their relatives. Similar antibodies can be found in autoimmume connective tissue disorders, allergies, infections and pregnancy.

Cell mediated immunity in the form of cytotoxic lymphocytes has been observed in up to a third of pernicious anemia patients.

CLINICAL MANIFESTATIONS

Mode of Onset and Initial Symptoms

By the time the patient seeks medical advice, the anemia is usually moderate in severity. Sometimes, the initial symptoms may be referable to the neurologic system and subsequently symptoms of anemia also supervene.

The triad of weakness, sore tongue and paresthesias is the classic symptoms complex at presentation but the initial symptoms actually vary. Often, they are suggestive of some digestive disorders; cardiac, renal, or genitourinary disease; or even mental aberration or an obscure infection.

GENERAL APPEARANCE

When the anemia is severe, the patient's skin has a delicate lemon-yellow tint resulting from the combination of pallor and mild icterus. The sclerae may be yellowish, but often the increased bilirubin concentration detectable in the blood is rarely manifested clinically. Diffuse or blotchy, brownish pigmentation may be interspersed with patches of vitiligo. Skin hyperpigmentation has been noticed in blacks. The skin may be dry, but often is peculiarly velvety and smooth, yet inelastic. Petechiae are unusual. Rarely, a change in

hair color, reversible by vitamin B_{12} treatment, has been found. Weight loss can be from slight to considerable (explainable on account of anorexia).

Fever

Hyperthermia of several degrees is common when anemia is severe. It disappears promptly following treatment.

Gastrointestinal System

Glossitis is common and painful. The tongue appears beefy red. Its entire dorsum may be involved, or red patches may be evident at the margins or on the dorsum. Rarely the entire mouth may be involved, causing burning and pain on swallowing. More frequently, patients experience only burning or soreness, particularly of the anterior half of the tongue. The intensity of glossitis often subsides but the soreness recurs at variable intervals. Between attacks, the epithelium of the tongue is left devoid of papillae, thus producing the smooth, glazed tongue that is characteristic of pernicious anemia. Partial loss of taste may follow. With adequate therapy, some restoration of the papillae often occurs. Loss of appetite is a frequent complaint. Diarrhea may be found in some patients. Patients may have anorexia, nausea, flatulence, a sense of fullness and epigastric discomfort, heartburn, vomiting and irregular abdominal pain of varying intensity. Attacks of paroxysmal pain resembling the gastric crisis of tabes dorsalis sometimes occur. Such attacks may be associated with vomiting and some abdominal rigidity. The pain may be symptomatic of changes in the spinal cord.

Cardiovascular and Genitourinary Systems

Cardiovascular symptoms of anemia have been discussed elsewhere (see under Red Blood Cells: Pathophysiology of Anemia, Cardiovascular Adjustments and Cardiorespiratory System in Chapter 3). In patients with severe neurological disease, impaired micturition and urinary retention may occur, predisposing them to infectious complications such as cystitis. Infertility has been ascribed occasionally to pernicious anemia.

Nervous System

With present day better diagnostic tools, severe symptoms referrable to neurologic system are not so frequently seen. The degree of nervous system involvement does not correlate well with the degree of anemia. Nor is neurologic disease a "late stage" of deficiency. Up to 25% cases of cobalamin deficiency present with neurologic symptoms rather than anemia. In such cases the only hematologic abnormality found is hypersegmentation of nuclei of neutrophils. If treatment is delayed the neurologic symptoms may progress while hematocrit may not change. The neurologic illness chiefly affects the white matter of the dorsal and lateral columns of the spinal cord and the cerebral cortex. Myelin degeneration and loss of nerve fibers in the dorsal root ganglia are found on histologic examination. Peripheral nerve degeneration also occurs, but whether this represents a distinct lesion or process secondary to spinal cord disease is not sure. Degeneration of the celiac ganglia and of Auerbach's and Meissner's plexuses, as well as lesions in the lateral horns of the spinal cord, have been noticed. Because multiple nerve pathways are involved, the terms subacute combined degeneration and combined system disease have been employed.

Subjective sensory complaints constitute the earliest and most frequent evidence of nervous system involvement. Most commonly, the patient experiences symmetric tingling or "pins and needles" sensation, first in the tips of the toes and later in a stocking-and-

glove distribution in all four limbs. Less often, the abnormal sensations may be described as numbness, coldness, or tightness. When paresthesias are the only symptom, little or no objective evidence of neurologic disease may be apparent.

In a more advanced stage, evidence of dorsal column involvement is clearer. The patient may complain of clumsiness, and his or her gait may be uncoordinated and ataxic. Reduced vibration sense, especially at higher frequencies is one of the earliest and most common objective signs (e.g. 256 cycles/sec). This abnormality often extends to the knees and, in severe cases, may go up to the iliac crest. Position sense may be impaired. The reaction to Romberg test may become abnormal.

The lateral columns (pyramidal tracts) become involved in the later and more severe stages of the disease. Weakness and a spastic or "scissors" gait may develop. The Babinski sign in the earliest and most reliable objective evidence of lateral column disease. Most commonly, tendon reflexes are substantially reduced, presumably because of peripheral nerve involvement, but hyperreflexia and clonus also may be observed as manifestations of pyramidal tract involvement.

Depression and impaired memory are among the most common mental symptoms but only the latter seem clearly related to vitamin B_{12} deficiency. With severe anemia, there may be a clinically demonstrable reduction in the level of consciousness, for which the most valuable single test is the serial subtraction of numbers such as 9 from 90 (81). Uncommonly, serious mental changes, as indicated by delusions, hallucinations, maniacal outburst, and paranoid and schizophrenia, may occur. Other less common neurological manifestations of pernicious anemia include ophthalmoplegia, atony of the bladder, impotence, orthostatic hypotension related to autonomic neuropathy, perversion or loss of the senses of taste and smell and retrobulbar neuritis (this may be aggravated or precipitated by the use of tobacco).

ERYTHROCYTE KINETICS, IRON AND BILIRUBIN METABOLISM

- RBC survival is reduced from 120 days to an average of 50 days (27–75 days)
- Chromium half-disappearance times become 16–21 days (normal, about 32 days)
- Excessive red cell destruction occurs because of red cell defect itself.
- Intravascular destruction occurs, in serum, haptoglobins are absent and methemalbumin may be present.
- Serum iron concentration is moderately increased unless a complicating iron deficiency is involved. Plasma total iron binding capacity may be reduced.
- Bone marrow sideroblasts and iron stores in macrophages also tend to be increased.
- Ferrokinetic studies reveal a threefold increase in the plasma iron-transport rate. Red cell utilization is markedly reduced, however, so that erythrocyte iron turnover is about normal. The data reflect ineffective erythropoiesis (Table 6.2).

Table 6.2: Ferrokinetic studies in normal and megalobastic anemia patients

	Normal individuals	*Patient with parnicious anemias*
Plasme iron µg/dL	105	152
Iron clearance (t ½, minutes)	86	46
Plasme iron transport (mg/day/dL)	0.7	2–4
Red cell utilization (%)	80	28
Erythrocyte iron turnover (mg/day/dL)	0.56	0.62

- As a result of increased production of bile pigment, slight unconjugated (indirect) hyperbilirubinemia may be observed, but normal values of serum bilirubin are common and values exceeding 2.0 mg/dL are unusual.
- Cytogenetics: A wide variety of chromosomal anomalies have been described in marrow cells from patients with megaloblastic anemia. Individual chromosomes may be elongated, and the frequency of randomly distributed gaps and breaks is increased. Separation or spreading of centromere has been noticed. The chromosomal abnormalities disappear promptly with effective treatment.

Epithelial Changes

Resemble changes as observed in marrow and red cell precursors. The cells and their nuclei are abnormally large, nuclear chromatin is less coarse than normal and multiple nuclei may be found. Such abnormalities can be seen in cells from the buccal mucosa and vaginal mucosa; the later shows atypical changes as observed in neoplasia. Gastric lavage cells show creased or folded nuclear membranes, and small aggregates of chromatin stand out on the relatively empty background of the nucleus. The cytoplasm, as well as the nucleus, is enlarged and has a more vacuolated and granular appearance than is normal.

Gastrointestinal Secretion

- The total volume of gastric secretion is reduced to an average of 15 mL (about 10%) of normal. Absence of hydrogen ion secretion (achlorhydria) with maximal histamine stimulation (40 µg/kg body weight) is an almost universal finding. Hydrogen ion secretion may be said to have occurred when the pH falls by at least one unit and to a value below 6.0 with such stimulation.
- The hallmark of pernicious anemia is the extreme deficiency of intrinsic factor in the gastric secretion. Gastric juice pepsin and pepsinogen levels in urine or serum tend to be reduced. Serum gastrin levels are increased in over 60 percent of cases with pernicious anemia, probably because gastrin secretion in normal subjects is inhibited by gastric acidity. The atrophic gastritis in patients with high gastrin levels tend to be limited to the body of the stomach and to spare the antrum. Excess plasma gastrin tends to induce hyperglycemia and to potentiate insulin secretion.

Serum LDH and Other Enzymes

Lactate dehydrogenase (LDH) activity is increased in most patients with megaloblastic anemia. The magnitude of the increase is related to the degree of anemia.

Serum LDH also may be increased in association with hemolytic anemia, but usually to a lesser degree than with megaloblastic anemia. LDH1 and LDH2 are increased most (from among the five isoenzymes). In pernicious anemia, LDH1 activity exceeds that of LDH2, whereas the converse is true of other hemolytic anemias. In megaloblastic anemia, the source of the serum enzyme is thought to be the cells destroyed by ineffective erythropoiesis in the marrow. With specific therapy, serum LDH levels return to normal within 1–2 weeks. Other enzymes increased are—malate dehydrogenase, 6-phosphogluconate dehydrogenase, and α-hydroxybutyrate dehydrogenase are increased to about the same extent as LDH; those for aldolase, phosphohexose isomerase, and isocitrate dehydrogenase are increased less consistently and to a lesser degree. Serum alkaline phosphatase activity is decreased in patients with pernicious anemia, as is that of serum and red cell cholinesterase.

OTHER BIOCHEMICAL FINDINGS

Serum potassium concentration may be mildly diminished in pernicious anemia. Negative nitrogen balance usually is observed during relapse. Slightly excessive urinary excretion of taurine and other amino acids may be noted, an increase in urinary hydroxyphenyl compounds, probably derived from tyrosine has been found. Conversion of formate to serine by lymphocytes is inhibited. The skeletal fraction of alkaline phosphatase activity in serum falls with deficiency and rises again with treatment. Cholesterol and phospholipid values in plasma are reduced and return to normal with treatment.

COURSE, PROGNOSIS, AND COMPLICATIONS

Present methods of treatment have completely changed the outlook in pernicious anemia. The most important present day cause of relapse is the patient's reluctance to continue treatment for life. Relapse is a common finding when the onset of some intercurrent disease or the need for some surgical measure distracted attention from antianemic therapy and caused it to be neglected at a time when it was most needed. Maintenance therapy should always be emphasised. The most serious long-term complication of pernicious anemia is carcinoma of stomach (incidence is about 3%). These patients also have higher incidence of colorectal carcinoma, especially during the 5-year period immediately following diagnosis. Upper intestinal carcinoid tumours also occur with increased frequency, possibly as a result of excessive gastrin secretion with consequent stimulation of enterochromaffin-like cells.

OTHER CONDITIONS ASSOCIATED WITH VITAMIN B_{12} DEFICIENCY

Dietary Deficiency of Vitamin B_{12}

This is unusual in nonvegetarians, as vitamin B_{12} has widespread distribution in foods of animal origin and its extensive storage in liver. In strict vegetarians (as in India) vitamin B_{12} deficiency can be found. Vegetarian diets are usually high in folic acid content. Mother's milk contains scanty amount of vitamin B_{12}. As dietary insufficiency is not so common, deficiency of vitamin B_{12} usually is the result of defective absorption of the vitamin. This situation may arise because of lack of Castle's intrinsic factor or because of various disorders affective the small intestine.

Gastric Surgery

During total gastrectomy, all intrinsic factor-secreting cells are removed, and vitamin B_{12} deficiency inevitably supervenes if necessary supportive therapy is not given. Partial gastrectomy can also lead to megaloblastic anemia, though some IF-secreting cells remain intact. Such a situation arises usually, often surgical treatment (subtotal or partial gastrectomy) conducted for gastric ulcer (in gastric ulcer more gastric tissue is removed as compared to surgery conducted for duodenal ulcer). Achlorhydria associated may also produce iron deficiency anemia or when combined, produces dimorphic anemia. Iron deficiency anemia often complicates the picture and may obscure the megaloblastic changes. About a third of cases treated with gastric bypass operations for morbid obesity develop low serum vitamin B_{12} levels within 2 years. Although gastric bypass does not interfere with IF secretion, it allows the IF to be exposed to pepsin and trypsin before binding of vitamin B_{12} takes place (binding makes IF resistant to proteolysis). Destruction of gastric mucosa following ingestion of corrosive materials can also lead to IF deficiency on account of chemically induced ablation of gastric mucosa.

Intestinal Disorders

Small Bowel Bacterial Overgrowth

Normally, bacterial overgrowth in the small intestine is prevented by gastric acid secretion and by the mechanical cleansing action of normal peristalsis. Any anatomical abnormality leading to stasis or recirculation of intestinal contents is likely to be accompanied by proliferation of microorganisms. Examples include diverticuli, anastomoses, fistulas, blind loops and pouches, strictures, scleroderma and amyloidosis. The bacterial population developing under such circum-stances resembles that of the colon (The promixal small bowel in healthy subjects may be sterile, or it may contain relatively low concentrations less than 10^4 organisms/mL of contents, of gram-positive aerobes or facultative anerobes, such as lactobacilli and enterococci along with some coliforms). In conditions mentioned above, anerobic lactobacilli and bacteroides tend to predominate, and coliforms and clostridia may be found in high concentrations. The number of organisms may be as much as 10^{10}/mL.

The main manifestations of the bacterial overgrowth syndrome are weight loss, megaloblastic anemia, steatorrhea and diarrhea. The steatorrhea probably results from bacterial hydrolysis of conjugated bile salts to form bile acids, which then are reabsorbed. By this mechanism, the concentration of intestinal bile salts becomes reduced below the critical level necessary for micelle formation, leading to malabsorption of fatty acids and monoglycerides. Vitamin B_{12} absorption is uniformly reduced in the bacterial overgrowth syndromes; the defect is not corrected by administration of IF, but is corrected transiently by prior administration of certain antibiotics, especially tetracyclines.

Metronidazole, lincomycin and cotrimoxazole are also effective. The absorptive defect results from successful competition by the bacteria for dietary vitamin B_{12}, thus making it unavailable to the host. Surgical correction of the anatomic abnormality leads to normalization of the bowel flora and restoration of normal vitamin B_{12} absorption.

Diseases Affecting the Ileum

As vitamin B_{12} is absorbed chiefly in the ileum, ileal resection or bypass or diseases that may be localized to the ileum lead to malabsorption of the vitamin. Vitamin B_{12} malabsorption has been observed in patients who have had as little as 1 foot of ileum resected and probably is always impaired if more than 6 feet are removed. The terminal ileum is affected in about 80 percent of patients with regional enteritis (Crohn's disease), and about 20–40% of these cases develop megaloblastic anemia. Both folate and vitamin B_{12} deficiency may occur in Crohn's disease.

Zollinger–Ellison Syndrome

Because of low pH of intestinal contents reaching the ileum, vitamin B_{12} absorption may be impaired in this disorder, however, megaloblastic anemia is rarely seen.

Fish Tapeworm Disease

Diphyllobothrium latum is a common parasite of freshwater fish and human infection results from ingestion of inadequately cooked fish. The deficiency of vitamin B_{12} results from impaired absorption. As in the bacterial overgrowth syndromes, the malabsorption probably results from competition between the worm and the host for dietary vitamin B_{12}. The tapeworm is able to take up and bind the vitamin firmly. The parasite lodges in the ileum in carriers without anemia, whereas in those with anemia, the site of attachment tends to be in the jejunum. Apparently, the latter location enables the worm to bind the

vitamin before it reaches the sites of absorption in the ileum. The anemia responds to expulsion of worms, but the response often is suboptimal if no vitamin B_{12} is given.

Tropical Sprue and Celiac Disease

These disorders lead to malabsorption of various nutrients. The most common cause of megaloblastic anemia in chronic malabsorption states is folic acid deficiency though.

Drug-induced Vitamin B_{12} Malabsorption

Para-aminosalicylic acid (PAS) if consumed for more than 6 weeks leads to moderate degree of vitamin B_{12} malabsorption. The absorptive defect disappears about 2 weeks after use of the drug is discontinued. Reversible malabsorption of vitamin B_{12} has also been observed in patients taking colchicine, neomycin, ethanol, omeprazole, or potassium chloride. Long-term usage of proton-pump inhibitors (PPIs) with attendant atrophic gastritis can also lead to vitamin B_{12} deficiency.

Familial Selective Vitamin B_{12} Malabsorption (Imerslund Syndrome, Imerslund–Gräsbeck Syndrome)

This inherited illness is characterized by the onset of megaloblastic anemia in childhood, usually during the first seven years of life, and by persistent proteinuria. It appears to be inherited as an autosomal recessive trait in most families. Patients with disorder are unable to absorb vitamin B_{12} but the proteinuria persists, perhaps because of an associated renal tubular defect. In heterozygotes, defects in vitamin B_{12} absorption lesser in severity than in homozygotes have been reported, but anemia does not develop. It is likely that the illness is caused by defective ileal receptors for the vitamin B_{12} intrinsic factor complex, or their lack.

Functionally Abnormal Intrinsic Factor

Abnormal intrinsic factor molecules have been found in some patients. These patients have an immunologically identifiable intrinsic factor in their gastric juice but the factor either cannot bind to B_{12} normally or the complex cannot bind to the ileal receptor. Such patients resemble cases of pernicious anemia in many respects, but their gastric juice is normal and autoantibodies to parietal cells and intrinsic factor are lacking. It responds completely to parenteral administration of vitamin B_{12}.

Chronic Pancreatic Disease

Cobalamin malabsorption appears to result from lack of pancreatic proteases and consequent inability to partially degrade the R proteins of saliva, gastric juice, and bile that compete with intrinsic factor. Nevertheless, fully developed megaloblastic anemia is rare in association with pancreatic disease.

Hemodialysis

Serum vitamin B_{12} concentrations fall progressively in patients undergoing maintenance hemodialysis. Parenteral administration correct the defect.

HIV Infection

Subnormal serum vitamin B_{12} levels have been noted in 20–30% cases of AIDS and are especially more common in individuals receiving zidovudine therapy. Hematologically neutrophilic hypersegmentation is found. Cobalamin absorption appears to be impaired, but a clear explanation for the abnormality is not found.

ETIOLOGY (FIG. 6.1)

1. Reduced intake—nutritional deficiency
2. Strict veganism
3. Impaired absorption

Gastric Cause

- Adult pernicious anemia
- Congenital lack of IF
- Total or partial gastrectomy
- Ingestion of corrosive materials.

Intestinal Cause

- Chronic tropical sprue
- Intestinal stagnant loop syndrome, e.g. jejunal diverticulosis, blind loop, strictures.
- Scleroderma
- Crohn's disease and ileal resection
- Congenital selective malabsorption with proteinuria
- Fish tapeworm infestation
- Zollinger–Ellison syndrome
- Severe pancreatitis
- Celiac disease

Transport Protein Defects

- Hereditary lack of transcobalamin II
- Abnormal transcobalamin II
- Abnormal B_{12} binding protein.

Hemodialysis

- Therapy with metformin/phenformin.

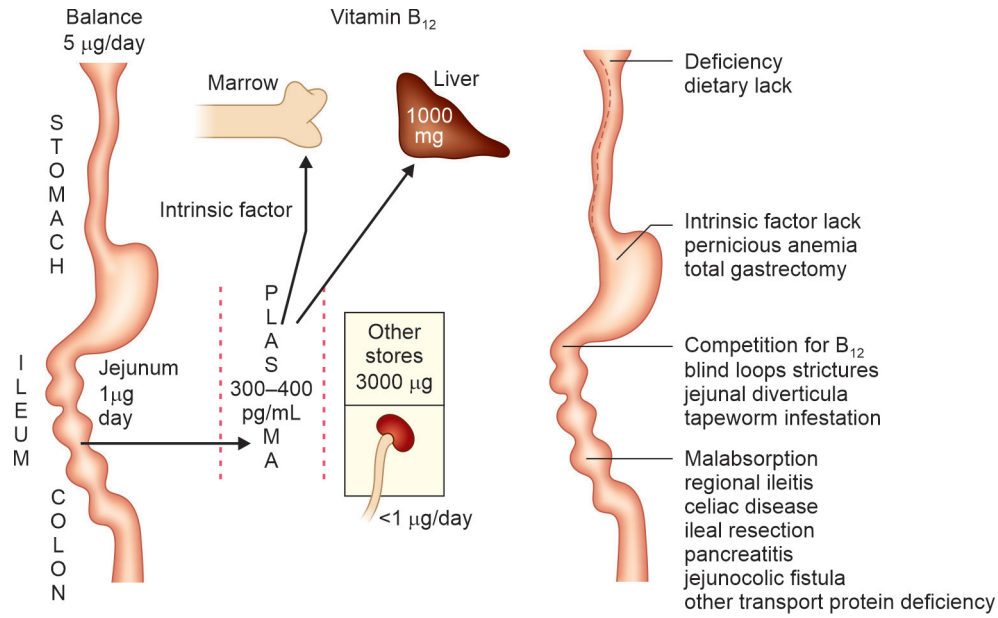


Fig. 6.1: Vitamin B_{12} balance and deficiency

CLINICAL MANIFESTATIONS

- Megaloblastic macrocytic anemia
- Glossitis
- Peripheral neuropathy and subacute combined degeneration of the cord.

SPECIAL TESTS FOR DIAGNOSING VITAMIN B_{12} DEFICIENCY

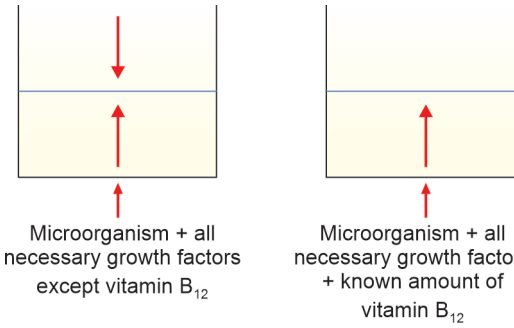
1. Serum vitamin B_{12} assay
2. Increased urinary excretion of methylmalonic acid
3. Radioactive vitamin B_{12} absorption test.

Serum Vitamin B_{12} Assay

- Microbiological assay
- Radioistope assay

Microbiological, Organisms Used

- *Euglena gracilis*
- *Lactobacillus leichmani*


Microorganism + all necessary growth factors except vitamin B_{12}
Microorganism + all necessary growth factors + known amount of vitamin B_{12}

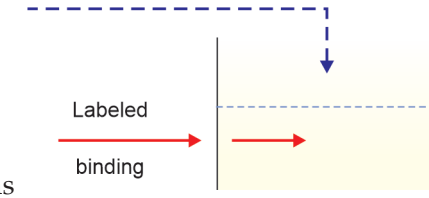
Test serum

- Both the tubes are inoculated and later the turbidity developed due to growth of the microorganism is measured and the serum vitamin B_{12} level deduced.
- Using *E. gracilis* normal values are 160–925 ng/L (mean = 475 ng/L)
- A value less than 100 ng/L implies frank megaloblastic anemia.

Radioisotope Assay

Principle saturation analysis.

1. Test serum
   +
   Vitamin B_{12} labeled with ^{57}Co
   Vitamin B_{12} binding protein or IF
2. Now separate free and bound forms
3. Compare with standards.
   - Normal values = 200–300 ng/L



Radioactive Vitamin B_{12} Absorption Test

Principle Ability to absorb ^{57}Co labeled vitamin B_{12} orally. If simultaneous administration of IF improves absorption, it implies lack of intrinsic factor.

Absorption of radioactive vitamin B_{12} can be measured in 5 ways:

1. Radioactivity in feces
2. Radioactivity in urine (Schilling test)
3. External counting over liver (chief storage organ)
4. Whole body counting.
5. Estimation of plasma radioactivity.

Schilling Test

- Give 1 mg unlabeled vitamin B_{12} parenterally
- Give 1 µg labeled vitamin B_{12} orally
- Within 24 hours one-third of absorbed radioactive vitamin B_{12} is flushed out into urine

- Normal excretion >10% of oral dose
- Pernicious anemia patients excrete <5%
- If it is abnormal: repeat the said procedure with simultaneous oral administration of IF
- If excretion increases, it implies lack of IF, If it does not then there is some absorptive defect (Repeat test to be done 48 hours later).

Variant of Schilling Test

- Give orally
  - Free ^{58}Co labeled vitamin B_{12} and
  - ^{57}Co labeled IF bound vitamin B_{12}
- Estimate amounts in urine
- If ^{57}Co labeled vitamin B_{12} is excrted more, there is intrinsic factor deficiency.

MANAGEMENT

Firstly identify the cause and type of deficiency. Replenish the deficient substance with adequate amounts and over a sufficiently long period. Avoid giving wrong vitamin or a mixture of agents. In addition to therapy with vitamin B_{12} or folate, it is sometimes possible to treat the underlying disease, for example, by surgical correction of anatomic lesions leading to small bowel bacterial overgrowth, expulsion of the fish tapeworn, or discontinuation of drugs leading to vitamin malabsorption.

Blood transfusions are rarely required in association with megaloblastic anemia, except when the anemia is severe. Specific therapy usually brings about a dramatic response in 48–72 hours. The most important indications for transfusion are signs of circulatory collapse, high output cardiac failure with pulmonary congestion at rest, or even severe intractable angina.

When administering vitamin B_{12}, several objectives should be kept in mind: (a) to supply vitamin B_{12} by a route whereby it will be absorbed, (b) to give amounts that will not only correct the deficiency of vitamin B_{12} but also will provide the patient with ample stores of the vitamin, having due regard for its excretion, (c) to observe carefully the effects of administration, thereby confirm the diagnosis and to discover associated deficiencies, (d) to ensure administrtion of vitamin B_{12}, in pernicious anemia, for life. Cyanocobalamin and hydroxocobalamin are given most often and are capable of inducing and maintaining remissions in vitamin B_{12} deficiency states, and both are nontoxic, except for the rare allergic reactions.

Parenteral injections of as little as 1 μg/day of vitamin B_{12} induce a complete hematologic remission in patients with pernicious anemia. If stores are also to be replenished, considerably larger dosages are required. Various regimens are available for parenteral administration. One regimen involves giving 100 mg of cyanocobalamin intramuscularly daily for 6 or 7 days and following up with daily reticulocyte counts. If clinical improvement is noted within a week, the same amounts are given on alternate days for seven doses, then every 3–4 days for 2–3 weeks, thereby producing a total of 1.8–2.0 mg B_{12} in 5 or 6 weeks. Alternatively, only 5 weekly injections of 1 mg each of hydroxocobalamin may be given, depending upon the desirability in the individual case for the patient to return for medical supervison. Under exceptional circumstances vitamin B_{12} can be given orally. Injectable preparations, however, can be given orally in fruit juice. Vitamin B_{12} can be given by the intranasal route but further studies are being awaited for recommending this route.

In patients with pernicious anemia, optimal maintenance therapy with cyanocobalamin consists of monthly injections of 100 mg for life. In other forms of megaloblastic anemia, this schedule may not be necessary, if the underlying cause can be corrected.

Response to Therapy

Symptomatic improvement often is recognised before any change in the blood is noticed. The patient becomes more alert and more co-operative, and appetite improves.

An increase in number of reticulocytes is the earliest and most useful sign of hematologic response to therapy. Reticulocytes count is highest on days 5–8 post-therapy commencement. Nucleated red cells may appear in the blood when the reticulocyte response occurs. After a delay of 5–7 days a perceptible increase in the VPRC is observed. Normal values are reached within 4–8 weeks regardless of the initial degrees of anemia

Mean corpuscular volume (MCV), after an initial rise owing to increased numbers of reticulocytes, gradually decreases, often in a biphasic pattern. The neutrohilic leukocytes increase in number, usually reaching normal values within a week. During the increasing number phase, few myelocytes or very rarely myeloblasts may be seen.

Hypersegmentation is not observed two weeks after commencement of therapy. Platelet count returns to normal within a week.

In bone marrow, ineffective erythropoiesis is reversed within 24 hours. After commencement of correct therapy, within 6–10 hours, the number of megaloblasts may decrease greatly. Within 24–48 hours, normoblastic maturation is seen. Giant metamyelocytes take about a week to disappear from bone marrow after commencement of therapy.

Biochemically, the increased plasma iron concentration characteristic of megaloblastic anemia in relapse decreases over 24–48 hour period. The serum iron level may remain low for several weeks. The plasma bilirubin concentration falls at about the end of the first week and is usually normal at 3 or 4 weeks. Urinary urobilinogen values begin to decrease at the end of the recticulocyte response. Free erythrocyte protoporphyrin levels increase with therapy.

The serum urate level increases as reticulocytosis occurs, presumably because of accelerated turnover of DNA in rapidly proliferating erythrocyte precursors. Urinary phosphorus content decreases rapidly when the appropriate vitamin is given, increases during reticulocytosis, and then gradually returns to normal values. Serum potassium concentration falls an average of 0.9 mEq/L during the first 48 hours of therapy and then gradually returns to normal over the next several weeks serum LDH levels return to normal within 1–2 weeks.

Neurologically, an occasional patient may experience a transient increase in paresthesias during the first week of treatment but this subsides and progressive improvement follows. Depending upon the duration and intensity of neurologic symptoms, full recovery may or may not take place. As a thumb rule, manifestations persisting a year after commencement of treatment may be considered irreversible. Absence of anemia conveys a worse prognosis for neurologic response.

Treatment (Basic Principles)

- In the absence of IF deficiency and malabsorption; oral administration of vitamin B_{12} is sufficient or else it can be given parenterally
- Vitamin B_{12} therapy improves all symptoms
- Folic acid administration alone will improve blood and tongue manifestations, the nervous system manifestations may be precipitated further.

FOLATE DEFICIENCY

The hematologic manifestations of folate deficiency and vitamin B_{12} deficiency are identical, namely megaloblastic anemia. No hematologic finding can reliably distinguish between the two deficiencies. They differ considerably, however, in their clinical settings. Folate deficiency occurs most often in individuals who are malnourished, usually because of their socioeconomic backgrounds. Traditionally, neurologic manifestations have been considered to be a major clinical difference between vitamin B_{12} and folate deficiency. Few reports, have however, indicated that polyneuropathy and subacute combined degeneration of the spinal cord, can occur in folate deficiency. In addition, certain neuropsychiatric symtpoms, e.g. depression, have also been reported. Folate deficiency has also been implicated in dementia. S-adenosylmethionine is essential to normal neurologic function and methionine synthesis requires both folate and vitamin B_{12} cofactors.

Causes of Folate Deficiency

Dietary Deficiency

A short period of dietary deprivation can lead to folate deficiency in an otherwise healthy individual. Normal folate stores remain adequate for only 2–4 months after the institution of a deficient diet, and in this respect folate differs markedly from vitamin B_{12}, the stores of which can last for several years.

Folate deficiency is seen more frequently in developing countries (main reason being poverty, but ignorance, customs, and religious tenets may also affect intake adversely). The diets associated with the development of folate deficiency are characterized by a predominance of starches and grains with relatively little animal protein or fresh, green vegetables. Nutritional folate deficiency has also been found in populations living in temperate areas. Major factors leading to folate deficiency in these regions are mental disturbances, chronic illness, alcoholism, food faddism, and poverty. Folate deficiency is often accompanied by other nutrient deficiencies as a part of a general picture of malnutrition. Particularly common is combined deficiency of vitamin C and folate because both vitamins are poorly stored and easily destroyed by heat used in food preparation. Often, treatment with both vitamins is necessary to completely correct megaloblastic anemia.

Alcoholism and Cirrhosis

Megaloblastic anemia in alcoholic patients usually is the consequence of folate deficiency. Serum folate levels are reduced, FIGLU excretion is increased and vitamin B_{12} levels are normal or increased. Dietary deficiency is the most important cause. Alcohol may also inhibit folate absorption, especially when folate stores are marginal, in part because chronic alcohol exposure inhibits an enzyme, pteroylglutamate hydrolase, that hydrolyses folate polyglutamates to the monoglutamate.

Alcohol also exerts direct toxic effects on bone marrow resulting in transient sideroblastic anemia. These two types of anemias often coexist after acute alcohol intoxication. Recovery from alcohol toxicity may lead to a reticulocytosis, which may suggest to the clinician that spontaneous recovery from anemia will occur, but if megaloblastosis persists, supplementation with folic acid will be required for full recovery.

Pregnancy

Folic acid is routinely given to all pregnant women these days, still, few pregnancies are complicated by megaloblastic anemia of pregnancy. Multiparity, adolescent pregnancy

and closely spaced, successive pregnancies are risk factors. Increased folate requirements combined with poor dietary intake is the usual cause.

Infants and Children

Megaloblastic anemia in infants and children is widespread where malnutrition is common, for example, where kwashiorkor is endemic.

The normal requirement for folate in infancy is about 50 μg/day. Normal human milk and raw or pasteurized cow's milk contain about 50 μg of folate per liter. Thus with normal rates of milk intake, the infant requirements for folate can be met with milk alone. Boiling, however, leads to a 40–80% reduction in the folate content of milk, and folate is partially destroyed when preparing powdered milk. In general, serum folate levels are higher in breastfed than in bottle-fed infants. Nevertheless, megaloblastic anemia has been reported in breastfed infants, possibly because of decreased amounts of folate in the milk of mothers with marginal folate stores.

Goat's milk contains low amounts of folate (average, 6 μg/L). Goat's milk anemia is a form of megaloblastic anemia of infancy occurring in babies fed goat's milk exclusively.

Nutritional folate deficiency in infants has to be differentiated from congenital pernicious anemia, familial selective vitamin B_{12} malabsorption, celiac disease, inborn errors of DNA synthesis, and vitamin B_{12} deficiency in breastfed infants of mothers with subclinical vitamin B_{12} deficiency.

Rapid Cellular Proliferation

Folate deficiency with megaloblastosis has been observed as a complication of chronic hemolytic disorders (sickle cell anemia, thalassemia, hereditary spherocytosis, acquired autoimmune hemolytic anemia, drug-induced hemolytic anemia with G6PD deficiency, and paroxysmal nocturnal hemoglobinuria). Deficiency should be suspected when hemoglobin concentration and/or reticulocyte numbers fall from previously stable levels or when a new requirement for blood transfusion develops. Folate deficiency has also been observed in myelofibrosis, sideroblastic anemia, leukemia, multiple myeloma, and psoriasis. These disorders have in common a rapid proliferation of red cells, other marrow elements, or epithelial tissues. Presumably, the need for DNA synthesis, and therefore the need for folate, is increased under such circumstances. Often, however, other reasons for folate deficiency in the reported patients coexist, including pregnancy and deficient diet.

Congenital Folate Malabsorption

A very rare disorder, becomes evident in the first few months of life. In addition to the absorptive disorder, defective transfer of folate to the central nervous system associated with mental retardation and ataxia has been seen.

Drug-induced Folate Deficiency

Less than 1% of patients taking phenytoin (Dilantin), primidone (Mysoline), carbamazepine, and phenobarbital, alone or in combination, are reported to develop megaloblastic anemia. Although clinically important megaloblastic anemia is unusual, mild folate deficiency and macrocytosis without anemia are relatively common. Serum folate and RBC folate levels, both can be reduced. The exact mechanism of deficiency development is not very clearly understood. Theories to the effect that these drugs cause folate malabsorption or that they increase folate catabolism have been put forward. Megaloblastic anemia has also been reported in women taking oral contraceptives and patients receiving sulfasalazine for

inflammatory bowel disease. Low folate levels have been associated with the long-term administration of cholestyramine in the management of hypercholesterolemia. Triamterine has also been implicated with folate deficiency, possibly due to an effect of the drug on folate absorption.

Tropical Sprue and Gluten-sensitive Enteropathy

Both these disorders bring about a generalized state of intestinal malabsorption characterized by steatorrhea, weight loss, weakness and deficiency of a wide variety of nutrients. The morbid anatomy of both diseases is characterized by varying degrees of villous atrophy with loss of intestinal surface. The lesions of gluten-sensitive enteropathy are most severe in the proximal intestine, and the ileum is involved in only a few patients. The lesions of tropical sprue tend to be less severe but more extensive, the entire small intestine being affected. The diseases differ not only in their geographic distribution, but also in pathogenesis and response to specific modes of therapy.

Tropical Sprue

It is endemic in many tropical areas (India included). For reasons unknown, it has been found more commonly in adults rather than children. Travelers to endemic areas may acquire the disease, but usually only after stay of 1 year or more. It is almost certainly infectious in origin, although a single etiologic agent has not been identified. Most likely, the disease represents the consequences of persistent small bowel infection by any of several enterotoxigenic strains of coliform bacteria, especially Klebsiella pneumoniae and, to a lesser extent *E. coli* and *Enterobacter cloacae.* Three clinical stages of tropical sprue have been recognised: Initial abrupt onset of diarrhea, anorexia, abdominal distension, and extreme asthenia, sometimes accompanied by fever. After an interval of weeks to months, the second, or deficiency phase supervenes as the result of prolonged malabsorption and depletion of nutrients, weight loss, glossitis, stomatitis, hyperkeratosis and continuing weakness are prominent in this stage. The third phase, megaloblastic anemia, develops after about 6 months. Because, the intestinal lesion is generalized, absorption of both folate and vitamin B_{12} is impaired. In most individuals with tropical sprue, symptoms progress relentlessly unless specific therapy is initiated. Administration of folate (5 mg/day) not only induces hematologic remission, but also may bring about improvement in intestinal symptoms and pathologic lesions. About half the patients may require long-term administration of antibiotics. Complete recovery can be expected in patients who receive proper treatment.

Gluten-induced Enteropathy

It includes both, coeliac disease of children and nontropical sprue (idiopathic steatorrhoea) in adults. The illness represents a possibly inherited, abnormal reaction to gluten a water insoluble, glutamine rich protein fraction of wheat and other grains-and to a related substance gliadin. The exact mechamism of disease induction is not clearly understood, but immune mechanism and environmental factors appear to be involved. Most gluten-sensitive patients carry IgG antigliadin antibodies.

In addition to weight loss, abdominal distention, and steatorrhea, patients with gluten-induced enteropathy may develop negative calcium balance with hypocalcemia, tetany, and demineralization of bones, with consequent bone pain and pathologic fractures. They may also become deficient in vitamin K-dependent coagulation factors. The most common cause of anemia in children with coeliac disease is iron deficiency, but folate deficiency

also occurs in 10–40% of cases. About 90% of patients with adult form of the disease have folate deficiency, whereas only about 40% manifest vitamin B_{12} malabsorption, presumably because the ileum is often spared. Iron absorption is diminished and iron stores often are low, but the anemia rarely appears hypochromic until the megaloblastic defect has been rectified. With a gluten free diet, the blood returns to normalcy over a period of several months. In "temperate sprue", the patients manifest with megaloblastic anemia without other clinical avidence of malabsorption. Other findings related to tropical sprue and gluten-induced enteropathy include; (1) increase in total fat in stool, (2) reduction in serum carotene and vitamin A levels. A malabsorption pattern is observed on radiographs of the intestine. The diagnosis of gluten-enteropathy requires demonstration of a response to a gluten-free diet. Alternatively, when the diagnosis is difficult to establish, the patient may be challenged with an oral dose of 3–5 g of gluten, which in sensitive subjects, promptly precipitates increase in diarrhea and fecal fat loss.

CAUSES OF FOLATE DEFICIENCY (Fig. 6.2)

1. Nutritional

- Especially old age
- Poverty
- Scurvy
- Partial gastrectomy
- Goat's milk anemia.

2. Malabsorption

- Tropical sprue
- Celiac disease
- Partial gastrectomy
- Extensive jejunal resection
- Crohn's disease.

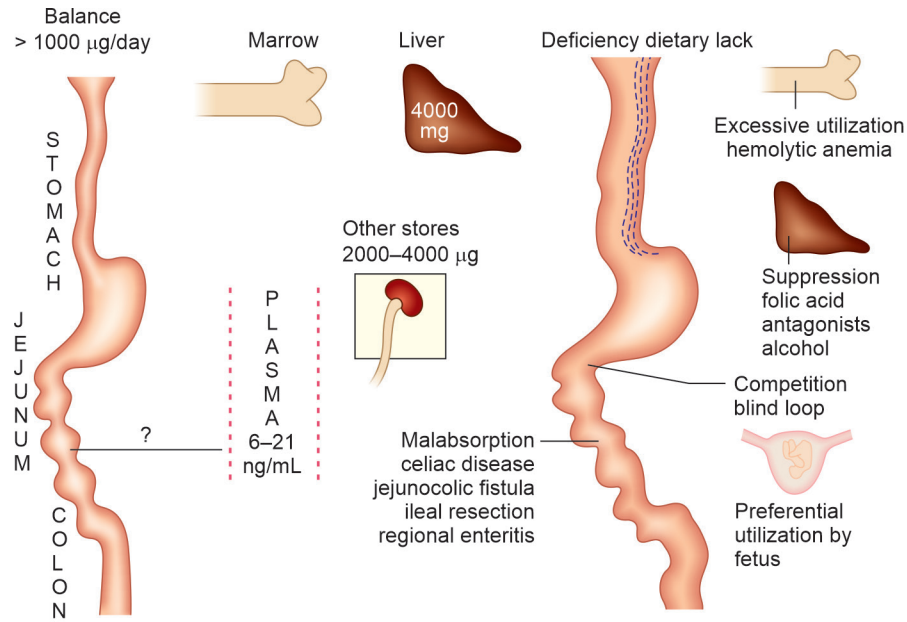


Fig. 6.2: Folic acid balance and deficiency

3. Increased Demand

a. Physiological

- Pregnancy
- Lactation
- Prematurity
- Infancy

b. Pathological

Hematological diseases

- Hemolytic anemia
- Myeloproliferative disorders
- Myelosclerosis
- Leukemia and lymphoma
- Sideroblastic anemia
- Multiple myeloma

Various carcinomas

- Inflammatory diseases
  - Crohn's disease
  - Tuberculosis
  - Rheumatoid arthritis
  - Psoriasis
  - Exfoliative dermatitis
- Hyperthyroidism

4. Excess Urinary Loss

- Active liver disease
- Congestive heart failure

5. Anticonvulsant Drug Therapy and Oral Contraceptives

6. Mixed

- Liver disease
- Alcoholism

7. Cytotoxic Drugs

CLINICAL MANIFESTATIONS

- Megaloblastic macrocytic anemia
- Glossitis
- Diarrhea, loss of appetite, malaise
- No nervous system symptoms.

SPECIAL TESTS FOR DIAGNOSING FOLATE DEFICIENCY

1. Serum Folate Assay

a. *Microbiological*
   - As for vitamin B_{12} except that *Lactobacillus casei* is used here
   - Normal value = 6–12 μg/L

b. *Radioisotope assay*
   - As for vitamin B_{12} except that cow's milk is used here as the binding protein
   - Value <4 μg/L implies megaloblastic macrocytic anemia (normal red cell folate = 160–640 μg/L).

2. FIGLU Test

Folate is essential for conversion of histidine to glutamic acid, formiminoglutamic acid (FIGLU) is intermediate product. In folate deficiency FIGLU is increased which appears in urine; this test, however, is not very specific.

3. Radioactive Folic Acid Test

Like Schilling test.

MANAGEMENT

The form of folate used most commonly for therapy is pteroylmonoglutamic acid, also known as folic acid. Dosage depends upon severity of disease. Folinic acid is equally effective.

Normal body folate stores are estimated at 5–10 mg, an amount sufficient for 2–4 months of normal hematopoiesis stores of this magnitude can be maintained in normal subjects by a dietary intake of 50–100 µg/day. The folates normally found in foodstuffs are polyglutamates, which must be converted to the monoglutamate before absorption. This conversion may be defective in many folate deficiency states, especially those associated with certain drugs and malabsorptive disorder. On the other hand, the absorption of pteroylmonoglutamic acid in patients with these disorders may be normal or near normal. In normal subjects, approximately 80% (range 40–95%) of orally administered folic acid is absorbed. When folic acid is administered in daily doses of less than 200 µg, little or none is lost in the urine. At higher doses, urinary loss is considerable.

Doses and Routes

Optimum hematologic response is elicited by 50–100 µg of folic acid given daily by the oral route. For replenishement of stores, however, larger amounts are required. A daily dose of 1 mg for 2–3 weeks should be enough. A patient who fails to respond to oral preparation will probably not respond to injections. Parenteral foms are required for patients who are unable to take medications by mouth.

Once stores are repleted, the need for maintenance therapy must be considered in the light of the underlying condition that produced the folate deficiency. If that condition can be corrected, normal dietary sources of folate should be enough. If this is not feasible, appropriate supplements in the range of 0–0.4 mg/day must be given. When the continued administration of diphenylhydantoin is necessary, a daily oral dose of 0.5–1 mg of folic acid may be needed. The usual dose of folinic acid is 3–15 mg, given intramuscularly. Folic acid is remarkably free of adverse reactions, except for a rare instance of allergic reaction. In some patients of epilepsy, seizures appear to have been aggravated by the administration of folate. By far the most important contraindication of folic acid therapy is the presence of an untreated deficiency of vitamin B_{12}. Here, a suboptimal hematologic response to folate may occur, but neurologic disease is not relieved. It is therefore advisable under such circumstances to perform vitamin B_{12} and folic acid assays.

PERIPHERAL BLOOD FINDINGS IN VITAMIN B_{12} OR FOLIC ACID DEFICIENCY (Figs 6.3 to 6.5)

- Anemia with macro (ovalo) cytosis
- Anisopoikilocytosis
- RBCs may show
- Howell–Jolly bodies

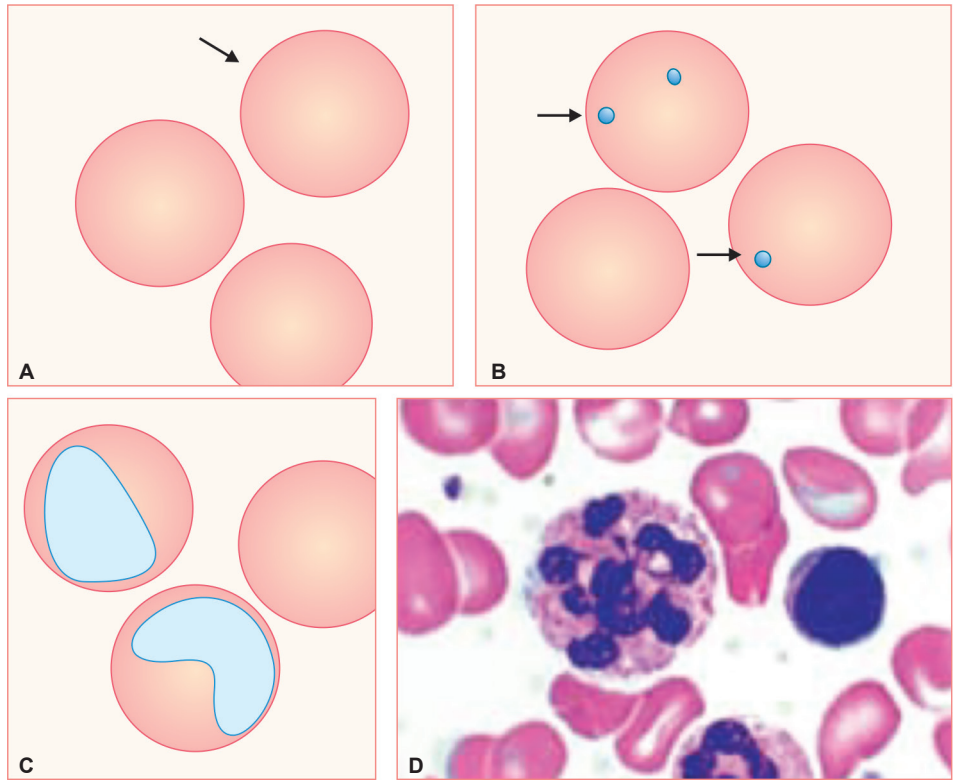


Fig. 6.3: (A) Basophilic stippling, (B) Howell–Jolly bodies, (C) Cabot rings, (D) Macro-ovalocytes and hypersegmented nucleus of neutrophil

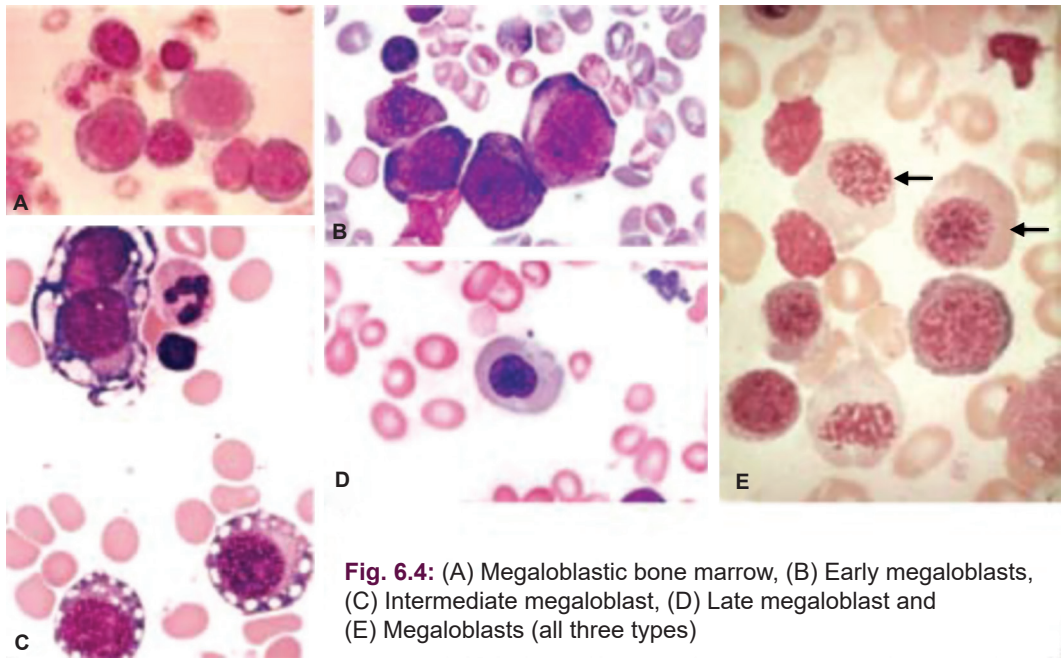


Fig. 6.4: (A) Megaloblastic bone marrow, (B) Early megaloblasts, (C) Intermediate megaloblast, (D) Late megaloblast and (E) Megaloblasts (all three types)

- Cabot rings
- Basophilic stippling
- MCV>96 fL (in mild anemia—it can be 100–110, and in severe anemia can go up to 160 fL)
- Moderate leucopenia due to neutropenia (TLC varies from 3,000–6,000 is because of absolute neutropenia)
- Hypersegmented neutrophils, i.e. more than 3 neutrophils having more than 5 nuclear lobes/100 neutrophils and increase in neutrophil myeloperoxidase
- High Arneth count
- Macropolycytes (large neutrophils)
- Mild, usually asymptomatic thrombocytopenia (so there is pancytopenia), especially if PCV is less than 25%, bleeding time may be prolonged, the blood clot may retract poorly and even purpura and retinal hemorrhages may occur.

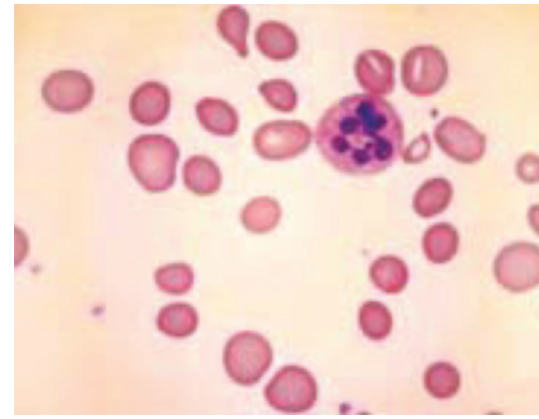


Fig. 6.5: Pernicious anemia, peripheral smear

BONE MARROW (Figs 6.4)

Dyserythropoiesis

Megaloblasts—a constant feature

- Increased cell size
- Nucleus becomes sieve-like
- Late megaloblast
  - has an eccentric indented/lobulated nucleus
  - may show Howell–Jolly bodies
- Dissociation of cytoplasmic, nuclear maturation (Hemoglobinization occurs faster than the nuclear maturation)
- Mitoses common, may be abnormal.
- Maturation arrest, promegaloblast and early megaloblasts constitute 50% of the erythroblasts.

WBC Series

- Large atypical cells
- Giant metamyelocytes
- Absolute number of granulocytes increases but is not evident because of simultaneous erythroid-hyperplasia.

Megakaryocytes

- Number is variable, occasionally diminished
- Have deep basophilic cytoplasm
- Hypersegmented nucleus.

MECHANISM OF ANEMIA IN MEGALOBLASTIC ANEMIA

- Bone marrow fails to respond to reduced RBC life span, hemolysis occurs both due to intracorpuscular and extracorpuscular causes (so serum LDH and bilirubin may rise)

- Maturation arrest—less production of cells (non-availability of DNA for cells, division stops)
  - Some die in bone marrow.
  - Others die in peripheral blood.

Biochemical Basis of Megaloblastic Anemia

Methotrexate, trimethoprim, triamterence and pyrimethamine block here (Fig. 6.6).

Vitamin B_{12} Deficiency Impairs Folate Availability

1. Methylfolate trap mechanism
2. Polyglutamate rather than monoglutamate is the active folate coenzyme. In vitamin B_{12} deficiency there is failure to synthesise polyglutamate coenzyme from monoglutamate. Hence DNA synthesis is impaired.

dU Suppression Test

- Short-term *in vitro* marrow cultures are used
- In normoblastic cultures, added deoxyuridine enters DNA thymine pathway and suppresses the subsequent incorporation of subsequently added tritiated thymidine into DNA.
- In vitamin B_{12} and folic acid deficiency the added deoxyuridine causes less suppression but the defect can be corrected by supplying the missing vitamin.

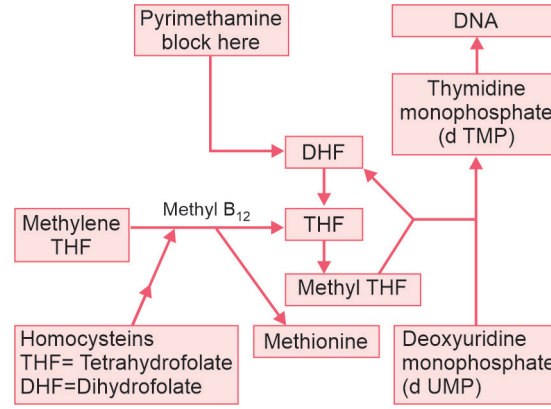


Fig. 6.6: Biochemical basis of megaloblastic anemia

DIAGNOSIS OF PERNICIOUS ANEMIA

SIGNS AND SYMPTOMS OF PERNICIOUS ANEMIA

Common to all Anemias

- Pallor
- Irritability
- Headache
- Fever
- Dyspnea
- Tachypnea
- Angina
- Heart failure
- Claudication.

Suggest Vitamin B_{12} or Folic Acid Deficiency

- Sore tongue
- Nonspecific gastrointestinal symptoms
- Flatulence
- Diarrhea
- Anorexia.

Suggest Vitamin B_{12} Deficiency

- Delirium
- Numbness and tingling of hands and feet
- Loss of fine learned movements
- Difficulty in walking
- Clumsiness
- Bladder and bowel dysfnction.

Suggest Pernicious Anemia

- Absence of histroy of malabsorption/starvation
- Genetic predisposition of Northern Europeans
- Premature graying of hair
- Vitiligo.

Diagnosis

- Clinical picture
- Macrocytic blood picture
- Megaloblastic bone marrow
- Histamine fast achlorhydria
- Low serum vitamin B_{12}
- Characteristic vitamin B_{12} absorption test
- Serum intrinsic factor and parietal cell antibodies.
- Reticulocyte response to vitamin B_{12} administration.

OTHER INVESTIGATIONS NEEDED IN FOLATE DEFICIENCY

- Jejunal biopsy: Celiac disease and tropical sprue
- Fat absorption test and flat GTT: Coeliac disease.

MEGALOBLASTIC ANEMIA UNRESPONSIVE TO THERAPY

- Lesch–Nyhan syndrome
- Orotic aciduria (orotic crystals in urine)
- Erythroleukemia.

INHERITED AND DRUG-INDUCED MEGALOBLASTIC ANEMIAS

Conditions associated with megaloblastic anemia, but without deficiency of vitamin B_{12} or folate, are considered here. These are discussed under two heading: inherited anomalies and disorders related to toxins and drugs.

INHERITED DISORDERS ALTERING DNA SYNTHESIS

These are rare inherited disorders and are probably inherited as autosonal recessive traits.

HOMOCYSTINURIA AND METHYLMALONYL ACIDURIA

There are at least 11 known different genetic disorders which are associated with methylmalonyl aciduria, homocystinuria, or both. They are often associated with pancytopenias. In some cases overt megaloblastic anemia has been observed. Some of these cases respond to large doses of vitamin B_{12}.

Intracellularly, cobalamin (Cbl) normally is converted to two coenzymes, methylcobalamin and adenosylcobalamin. Methylcobalamin is required for the conversion of homocystine to methionine, whereas adenosylcobalamin is a cofactor in the conversion of methylmalonyl coenzyme A to succinyl coenzyme A. The cobalt molecule in cobalamin that enters the cells is in the stable (3+) oxidation state and must be reduced to the labile (2+) state or (1+) state to be active as an enzyme cofactor. Of the 11 disorders (earlier written about), seven are disorders of mitochondrial cobalamin metabolism and are designated as Cbl-A to Cbl G respectively. In additon to these defects in cobalamin metabolism, methylmalonyl aciduria can result from complete (mut°) or partial deficiency (mut–) of the enzyme methylmalonyl coenzyme A mutase. Homocystinuria is also caused by cystathionine synthase deficiency and methylene tetrahydrofolate reductase deficiency (Neither is associated with any hematologic manifestations). Cbl A to Cbl G respond in varying degrees to large doses of vitamin B_{12}. Hydroxocobalamin is by and large more effective than cyanocobalamin and is given in a dose of 1 mg per day.

ABNORMAL VITAMIN B_{12} TRANSPORT

Transcobalamin II (TC II) is the physiologic transport protein for vitamin B_{12}. Absence of TC II or abnormal TC II molecules inherited as autosomal recessive traits can lead to megaloblastic anemia. Lack of the protein is associated with severe, potentially fatal disease, usually detected within the first 6–20 weeks of life. Signs include weakness, diarrhea, failure to thrive, megaloblastic anemia, pancytopenia, mucosal ulcers, and hypogammaglobulinemia. Neurological abnormalities may be present at onset or may develop later, a delay in treatment may make these abnormalities disabling. Several cellular and humoral immunologic deficiencies may be found.

Often, serum cobalamin levels are normal because most of the vitamin in plasma is bound to TC I and TC III. Homocystinuria or methyl malonic aciduria has occasionally been detected. The Schilling test for vitamin B_{12} malabsorption may be abnormal. Diagnosis depends on the demonstration of a lack of TC II by means of chromatography or radioimmunoassay. Therapy with physiologic amounts of vitamin B_{12} is ineffective, but large doses (1000 mg two to three times weekly) can reverse the clinical findings and bring about normal development.

OROTIC ACIDURIA

Orotic aciduria is a disorder of pyrimidine metabolism inherited as an autosomal recessive trait. It represents a defect in the synthesis of uridine 5′ monophosphate. Two enzymatic functions are defective in most patients: orotate phosphoribosyl transferase (OPRT) and orotidine 5′ monophos-phate decarboxylase (ODC). Both appear to be properties of a single, structural protein, uridine 5′ monophosphate synthase (UMPS). The gene for the enzyme is located on the long arm of chromosome 3. The onset of illness is noted in the first few months of life, but detection may be delayed for as long as 7 years. The patients have severe megaloblastic anemia and delayed physical and mental development.

They excrete large amounts of orotic acid in the urine. Orotic acid crystalluria may occur with associated hematuria and even urinary tract obstruction. Some patients may have associated cardiac malformations and/or defective immune function with increased susceptibility to infection. Deficient OPRT and ODC activities can be detected in erythrocytes and cultured fibroblasts. The condition does not respond to treatment with vitamin B_{12} or folate, but hematologic remission and restoration of growth and development

follow oral administration of uridine in doses of 100–200 mg/kg/day mental retardation and immune function may not be completely restored by uridine administration.

THIAMINE RESPONSIVE MEGALOBLASTIC ANEMIA

TRMA is a triad consisting of megaloblastic anemia, diabetes mellitus, and sensorineural deafness. It shares some features with a more common disorder known DIDMOAD (diabetes insipidus diabetes mellitus optic atrophy and dilatation of the urinary tract), which may also be associated with TRMA. It is in all probability a genetic disorder. Consanguineous parents have been reported in a majority of the patients, and the mode of inheritance is almost certainly autosomal recessive. Patients have been treated with high doses of thiamine, either thiamine hydrochloride 50–100 mg per day or S-benzoyloxymethyl (BOM) thiamine, 50 mg per day. With treatment, a prompt reticulocytosis and relief of anemia occurs, with relapse of treatment. In most patients, hyperglycemia is moderated, and insulin requirements may be reduced and even eliminated. Cardiac arrhythmias also respond promptly to treatment. Deafness may improve, but is often resistant to treatment.

OTHER POSSIBLE DISORDERS

1. Methionine synthase (methyl tetrahydrofolate transferase) deficiency
2. Dihydrofolate reductase deficiency
3. Glutamate formiminotransferase deficiency
4. Lesch–Nyhan syndrome.

DRUG-INDUCED DISORDERS OF DNA SYNTHESIS

Megaloblastic anemia is a toxic side effect of a variety of drugs used for chemotherapy of leukemias and solid tumors and for immunosuppression. This effect has been reported with the use of 6-mercaptopurine, 5-fluorouracil, cyclophosphamide, cytosine arabinoside, azathioprine, and the folate antogonists. As these drugs exert their beneficial effects by interfering with DNA synthesis, the toxic effect may be considered an extension of their therapeutic actions. Megaloblastic changes are signaled by the development of pancytopenia and by the appearance of hypersegmented neutrophils and oval macrocytes in the blood.

FOLATE ANTAGONISTS

Methotraxate and folate antagonists act by inhibiting dihydrofolate reductase. Less profound inhibition of this enzyme has been noticed as a side effect of drugs used for other purposes, including proguanil, pyrimethamine, triameterene, pentamidine and trimethoprim.

In combination with sulfamethoxazole, trimethoprim is a widely used antibiotic and the preferred drug for use in the treatment of *Pneumocystis carinii* pneumonia. It exerts its antimicrobial effects by inhibiting bacterial dihydrofolate reductase. Human dihydrofolate reductase is less sensitive to trimethoprim than is the bacterial enzyme, and in the usually therapeutic doses, hematologic side effects are rare; however, in higher doses acute megaloblastic anemia may ensue. Folinic acid, a reduced form that bypasses the dihydrofolate reductase step, rapidly reverses the hematologic effects of folate antagonists. When used with trimethoprim, antimicrobial activity is not compromised because microorganisms are unable to utilize folinic acid. Doses of folinic acid 15 mg/day have been recommended for treatment.

NITROUS OXIDE

The anesthetic gas nitrous oxide (N_2O) reacts with reduced cobalamins (Cbl-Co+1) to form inactive oxidised cobalamins (Cbl-Co+3). By this mechanisms, intracellular pathways that depend on cobalamin coenzymes are inhibited selectively. Folate metabolism is impaired secondarily. Megaloblastic anemia and/or neurologic abnormalities have been seen in patients exposed to nitrous oxide for treatment of tetanus, for analgesia related to open heart surgery or other surgical procedures, or as an adjunct to physiotherapy. Nitrous oxide anesthesia may precipitate neurologic disease in patients with subclinical vitamin B_{12} deficiency.

OTHER DRUGS AND TOXINS

Megaloblastic dysplasia has also been reported in association with arsenic intoxication and with chlordane exposure.

CAUSES OF MACROCYTOSIS OTHER THAN MEGALOBLASTIC ANEMIA

- Alcohol
- Liver disease
- Obstructive jaundice
- Myxedema
- Reticulocytosis
- Cytotoxic drugs
- Aplastic anemia
- Primary acquired sideroblastic anemia
- Pregnancy
- Myeloproliferative and myelodysplastic syndromes
- Respiratory failure
- Post-splenectomy
- Chronic obstructive pulmonary disease
- Hemolytic anemia
- Response to hemorrhage.

RECAPITULATION—APPROACH TO MEGALOBLASTIC ANEMIA

The anemia should be characterized. Pancytopenia and systemic impairment should be evaluated. The etiology of megaloblastosis should be identified.

Initial Studies

Initial workup for megaloblastic anemia should include the following assays:

- Complete blood count (CBC)
- Red blood cell (RBC) indices
- Peripheral blood smear
- Reticulocyte count
- Lactate dehydrogenase (LDH)
- Indirect bilirubin
- Iron and ferritin
- Serum cobalamin
- Serum folate, and possibly RBC folate

The LDH level is usually markedly increased in severe megaloblastic anemia. Reticulocyte counts are inappropriately low, representing lack of production of RBCs due to massive intramedullary hemolysis. These findings are characteristics of ineffective hematopoiesis that occurs in megaloblastic anemia as well as in other disorders such as thalassemia major.

PERIPHERAL SMEAR MORPHOLOGY

A peripheral smear may reveal macro-ovalocytes, a characteristic of megaloblastosis (Fig. 6.7). They should be distinguished from macrocytes that are not oval, which can occur in liver disease and hypothyroidism. Polychromatophilic macrocytes, reticulocytes, and immature RBCs can be seen in hemolytic anemia and disorders associated with increased RBC production.

Peripheral smear of blood from a patient with pernicious anemia. Macrocytes are observed, and some of the red blood cells show ovalocytosis. A 6-lobed polymorphonuclear leukocyte is present.

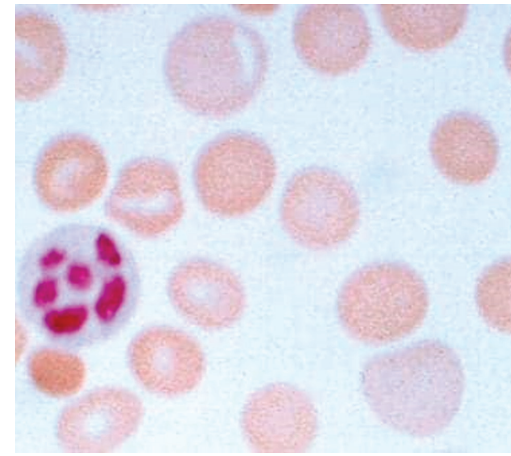


Fig. 6.7: Peripheral smear shows macro-ovalocytes

- Single and multiple Howell–Jolly bodies, nuclear fragment, may be seen in RBCs. Cabot rings, remnants of mitotic spindles, may also be present in RBCs.
- Nucleated RBCs and megaloblasts can be seen.
- Smears may reveal hypersegmented neutrophils if at least 5% neutrophils have 5 or more lobes. Normal neutrophils contain 3–4 lobes.
- Macrocytosis due to cobalamin or folate deficiencies may be masked in patients with iron deficiency. However, hypersegmented neutrophils can persist in iron deficiency.

Bone Marrow Aspiration

Bone marrow aspiration is usually not needed to make the diagnosis of vitamin B_{12} deficiency. However, it can help rule out myelodysplasia and assess iron stores. The bone marrow is hypercellular with erythroid hyperplasia. Erythroid precursors have megaloblastic features being larger than normoblastic cells. In addition, nuclear maturation is immature relative to cytoplasmic maturation. Megaloblastic changes are most prominent in more mature RBC precursors. Giant bands, neutrophil precursers, can be present. Megakaryocytes may be large and hyperlobulated .

Bone marrow megaloblastic changes are reversed within 12 hours after treatment with cobalamin or folate, and bone marrow morphology appears to be normal within 2–3 days. Therefore, bone marrow aspiration, if necessary, should be performed as soon as possible and preferably before therapy.

Primary Tests for B_{12} and Folate Deficiencies

Serum B_{12} (cobalamin)

Reference range: 200–900 pg/mL
- Borderline: 180–250 pg/mL
- Associated with anemia and neuropathy: <180 mg/L
- Diagnostic of B–12 deficiency: <150 mg/L

The serum cobalamin level can be normal in the following circumstances:
- In some inborn areas of cobalamin deficiency
- Transcobalamin II (TC II) deficiency
- Cobalamin deficiency due to nitrous oxide

Serum cobalamin levels may be low in the following circumstances:
- Pregnancy
- Oral contraceptives
- Transcobalamin I (TC I) deficiency
- Severe folic acid deficiency
- Patients taking large doses of ascorbic acid

Serum Folate

Folate reference range in adults: 2–20 ng/mL
- Folate deficiency likely (overlap with normal): <2.5 ng/mL

The following should be considered:
- Effect of diet: A single meal may falsely elevate serum folate levels to normal. Hence, blood should be drawn prior to transfusions, meals, and therapy to achieve accurate results.
- Hemolysis: Might cause false positive results

RBC Folate

Reference range for adults: >140 ng/mL
- Not affected by diet and reflects tissue stores (folate content is established early in RBC development)
- Affected by hemolysis
- Low in severe B_{12} deficiency
- Test is intricate and expensive

Serum for folate and cobalamin should be frozen and stored prior to meals or therapy if the tests cannot be performed within a reasonable time frame.

Lab Tests to Confirm and Distinguish B_{12} and Folate Deficiencies

Serum homocysteine and methylmalonic acid (MMA) levels are helpful confirmatory tests for cobalamin and folate deficiencies. Both are increased in cobalamine deficiency. Homocysteine but not MMA is increased in folate deficiency. Homocysteine and MMA levels should be used if the clinical presentation and serum vitamin B_{12} and folate levels are ambiguous.

The MMA level can be increased in the following circumstances:
- End-stage renal disease
- Inborn error of methylmalonic acid metabolism

Serum homocysteine can be increased in the following circumstances:
- Homocystinuria
- Hyperhomocysteinemia
- MTHFR C677T

Serum homocysteine can be decreased in the following circumstances:
- A high rate of conversion back to methionine
- Low production
- A high rate of conversion of into sulfite/sulfate, etc.

Intrinsic Factor (IF) Blocking and Parietal Cell and Antibodies

IF antibodies, type 1 and type 2, occur in 50% of patients with pernicious anemia and are specific for this disorder. Therefore, they can be used to confirm the diagnosis of pernicious anemia. Parietal cell antibody occurs in 90% of patients with pernicious anemia but can also occur in thyroid disease and other autoimmune disorders. Therefore, parietal cell antibodies are not specific for pernicious anemia.

A Valuable Test that is no Longer Available (Schilling Test)

The value of a Schilling test (a radiometric test) is that it can confirm B_{12} deficiency, can be done after patient has been given B_{12} therapy, and can distinguish between pernicious anemia and failure in transport or ileal uptake. Unfortunately, the test is no longer available at most hospitals. The 3 parts to the Schilling test are as follows:
1. First, radioactive cyanocobalamin is given orally and its urinary secretion is measured to estimate cobalamin uptake. Low urinary secretion suggests pernicious anemia, a failure in intestinal transport, defective uptake of cobalamin in the terminal ileum, or a blind loop syndrome.
2. The second part is performed in the same manner, except that IF is given orally along with radioactive cyanocobalamin. If IF restores cobalamin uptake, the patient most likely has pernicious anemia. If not, an abnormality in cobalamin intestinal transport, defective absorption in the terminal ileum, or a blind loop syndrome might be responsible for the deficiency.
3. In the third phase, the patient is treated with antibiotics before the administration of radioactive cyanocobalamin. If antibiotics restore cobalamin uptake, the patient most likely has a blind loop syndrome.

Diagnostic Therapeutic Trial

If the results of the evaluation are ambiguous, a clinical trial of the effects of cobalamin therapy may be indicated. However, a clinical trial of folate is contraindicated if cobalamin deficiency has not been ruled out. The administration of folate to patients with cobalamin deficiencies may precipitate or worsen neurologic impairment.

Other Studies

Predict the need for iron therapy since iron stores can be consumed during cobalamin or folate therapy.

Radiographic imaging of the upper and lower gastrointestinal tract may be useful for detecting abnormalities that could cause a blind loop syndrome. These procedures also may detect defects in the terminal ileum that might interfere with cobalamin absorption.
Other tests that may be considered include the following:
- With cobalamin deficiency, evaluate and rule out autoimmune disorders, Zollinger–Ellison syndrome, pancreatic insufficiency, fish tapeworm infestation, Imerslund–Grasbeck syndrome, Crohn disease, or ileal scarring.
- With folate deficiency, evaluate evidence for malnutrition and alcoholism, sprue, chronic hemolysis, and exfoliative dermatitis.

MEGALOBLASTIC ANEMIA TREATMENT AND MANAGEMENT

Approach Considerations

Once drug-induced megaloblastic changes and myelodysplasia-related megaloblastosis have been ruled out, most patients are treated with cobalamin or folate. Since megaloblastic anemias usually develop gradually, many patients adjust to low hemoglobin levels and do not require transfusions. Transfusion therapy should be restricted to patients with severe, uncompensated, and life-threatening anemia.

Go to *anemia, chronic anemia, myelophthisic anemia, hemolytic anemia,* and *sideroblastic anemias* for complete information on these topics.

Cobalamin Therapy

Cobalamin (1000 μg) should be given intramuscularly daily for 2 weeks, then weekly until the hematocrit value is normal, and then monthly for life. A dose of 1000 μg is large, but it may be required in some patients. The reader should be aware that several other protocols for cobalamin therapy have been recommended. It is important to emphasize that patients with mental and neurological impairment due to cobalamin deficiency should be treated more aggressively.

Oral cobalamin (1000–2000 μg) also can be administered. Oral cobalamin is less expensive and is better tolerated by patients. A wide range of doses and schedules have been recommended. For example, a randomized controlled trial in 40 vegans and vegetarians with marginal vitamin B_{12} deficiency found that adequate vitamin B_{12} levels could be achieved with cobalamin given in either a sublingual dosage of 50 μg/day (350 μg/week) or 2000 μg/week in a single oral dose.

Patients receiving oral dosages should be monitored for the desired response, since absorption can be variable and may be insufficient in some patients. Note the following:

- Oral cobalamin is indicated in patients with hemophilia to avoid bleeding from intramuscular injections.
- Intramuscular cobalamin and not oral cobalamin should be used to treat patients with cobalamin-related neurological disorders.
- One advantage of parenteral over oral cobalamin is that all abnormalities in cobalamin absorption are bypassed.
- It may be practical to administer parenteral cobalamin initially and then switch to oral cobalamin.
- Proton pump inhibitors, H_2-receptor antagonists, and metformin may reduce serum vitamin B_{12} concentrations by inhibiting the absorption of vitamin B_{12}.

Patients who have undergone either a total or partial gastrectomy should be started on replacement therapy after the surgery to prevent the development of megaloblastosis. Oral vitamin B_{12} supplementation is effective and safe in patients who underwent total gastrectomy and should be considered the preferential form of supplementation.

Folate Therapy

Folate should be administered orally. If this is difficult, comparable doses can be administered parenterally. In addition, the patient should consume a folate-enriched diet. The dosage range for folate is 1–5 mg daily; 1 mg/day is the usual dosage for adults with megaloblastic anemia, while a higher dosage is indicated in hemolysis, malabsorption, alcoholism, and exfoliative dermatitis. However, there is no harm in giving the higher dosage of folate.

Folate should be administered prophylactically during pregnancy, lactation, and the perinatal period. Folate is also indicated in patients with chronic hemolytic anemias, psoriasis and exfoliative dermatitis, and during extensive renal dialysis. Folate therapy has been recommended in patients with hyperhomocysteinemia who are at risk for thromboembolic complications.

Fortification of foods and folic acid supplements have been recommended to reduce the risk of pancreatic, cervical, and colon cancers. Folic acid supplements are indicated in patients with kidney failure. Folate supplementation is indicated in elderly persons. However, opponents of fortification and supplementation are concerned that giving folate-fortified foods to persons with unrecognized cobalamin deficiencies will increase the frequency of cobalamin-induced neuropsychiatric disorders.

The dosage and protocol for folic acid therapy and supplementation in the various disorders mentioned above are summarized in a communication from the Mayo Clinic.

Warning

Folate therapy should not be instituted in a patient with megaloblastic anemia if cobalamin deficiency has not been definitively ruled out. The danger is that folic acid will improve the anemia but not the neurological complications of cobalamin deficiency, and the neurological disorder will worsen. Both cobalamin and folate should be given if cobalamin deficiency has not been ruled out.

Monitoring Response to Therapy

Although patients may feel better as soon as therapy is started, improvements must be monitored. Laboratory tests to order include the following:

- Complete blood cell count
- Reticulocyte count
- Lactate dehydrogenase (LDH) level
- Indirect bilirubin
- Hemoglobin level
- Serum potassium level
- Serum ferritin.

Elevated levels of LDH and indirect bilirubin should fall rapidly. A prolonged elevation of the LDH level indicates a failure of therapy, development of iron deficiency, or an error in diagnosis.

Reticulocytosis should be evident within 3–5 days and peaks in 4–10 days. Leukocyte and platelets counts are usually restored to normal within days after therapy has been started, but hypersegmented neutrophils may persist for 10–14 days.

The hemoglobin should rise approximately 1 g/dL each week. This rise is valuable for monitoring a complete response. If the hemoglobin does not rise appropriately and is not normal within 2 months, other causes of anemia, such as iron deficiency, should be considered.

Serum potassium levels can fall during therapy for severe cobalamin or folate deficiency, which can lead to sudden death. Therefore, potassium should be monitored and supplements may be indicated.

Iron deficiency can occur in the course of treatment due to the consumption of iron stores for red blood cell production. The development of iron deficiency can impede the response to cobalamin or folate therapy. Iron therapy may be indicated

Treatment of Other Related Conditions

Other related conditions, if present, should be addressed as follows:

- Blind loop syndrome should be treated with antibiotics.
- Patients with transcobalamin II (TCII) deficiency may require higher doses of cobalamin.
- Tropical sprue should be treated with both cobalamin and folate.
- Acute megaloblastic anemias due to nitrous oxide exposure can be treated with folate and cobalamin.
- Fish tapeworm infection, pancreatitis, Zollinger–Ellison syndrome, and inborn errors should be treated with appropriate measures.

Dietary Measures

Patients with folate or cobalamin deficiency should receive dietary education on the choice of foods and instructions on how to prepare foods.

Patients should have diets rich with folic acid. Examples of such foods include asparagus, broccoli, spinach, lettuce, lemons, bananas, melons, liver, and mushrooms. To prevent loss of folate, foods should not be cooked excessively and should not be diluted in large amounts of water. To prevent cobalamin deficiency, vegetarians should include dairy products and eggs in their meals. Patients should know that goat milk contains little folate.

CHAPTER

7

Symptomatic Anemias

Considered in this chapter are anemias occurring in association with other well-defined systemic disorders. Since anemia constitutes symptom of these disorders and its severity is proportional to the severity of the underlying disease—they are referred to as symtopmatic anemias. The disorders that can be mentioned here are infection, renal failure, malignancy, liver disease, endocrine disorders, collagen disease protein malnutrition and scurvy. The anemia of acute hemorrhage is also discussed here as no primary hemotological abnormality causes it.

Most of the anemias occurring in conjunction with disorders mentioned are normocytic, normochromic; however, macrocytic or microcytic anemias also can sometimes occur. These anemias are not a consequence of iron, vitamin B_{12} or folic acid deficiency and, therefore, treatment with the same is useless. These anemias respond only to rectification of the causative disorder. Iron, however, is indicated in acute hemorrhagic anemia.

ACUTE HEMORRHAGIC ANEMIA

BLOOD PICTURE

- Leads to normocytic or sightly macrocytic normochromic anemia
- Hemoglobin and PCV values vary with the duration between hemorrhage and their estimation.
  Immediately afterward—
  — No change
  3 hours more or later —
  — Hemoglobin and PCV, both are reduced.
- 24–48 hours after the episode, first signs of regeneration are seen in the form of reticulocytosis, polychromatophilia, slight macrocytosis (younger RBCs are released).
- With severe hemorrhage, sometimes even normoblasts may appear in peripheral circulation.
- There is leukocytosis with neutrophilia (slight shift to the left may also occur, i.e. band forms or metamyelocytes may be seen on peripheral smear)
- In addition, there is mild thrombocytosis too.

HEMATOLOGIC FINDINGS

Immediately following a hemorrhage, the plasma volume and red cell mass are reduced in proportional amounts; therefore, no decrease is observed in the volume of packed red cells (VPRC). Because of the time required to effect restoration of the blood volume, the amount of blood loss tends to be underestimated by the degree of anemia, especially early

in the disease course. The VPRC may not reach the minimum value until 3 day or more after the hemorrhage ceases.

On first detection, the anemia is normocytic, normochormic with few signs of erythrocytic regeneration. Some increase in reticulocytes is usually perceptible within 3–5 days, and maximal values are reached at 6–11 days. Reticulocytosis is proportionate to degree of hemorrhage but rarely exceeds 15%. Other signs of erythrocyte regeneration include polychromatophilia and macrocytosis, and the MCV may transiently increase. If first seen at this stage, the finding may be confused with hemolytic anemia: however, signs of hyperbilirubinemia are conspicuously absent, unless the bleeding has occurred into a body cavity or tissue space.

During or immediately following hemorrhage, the platelet count, whole blood coagulation time, and plasma fibrinogen levels may be reduced. These abnormalities are usually transient and tend to disappear within 15 minutes after cessation of bleeding. Thereafter there is thrombocytosis within one hour, values as high as 10 lakh/mm^3 are observed. If severe shock develops, disseminated intravascular coagulation may complicate the picture.

Neutrophilic leukocytosis often follows hemorrhage, reaching maximal levels at 2–5 hours. Typically TLC is between 10,000 and 20,000 cells/mm^3 but may reach as high as 35,000 cells/mm^3.

CLINICAL ASPECTS

- These depend upon rate and amount of blood lost
- Blood volume reduction may lead to:
  - Weakness
  - Nausea
  - Fainting
  - Sweating
  - Ashen pallor
  - Hypotension and tachycardia
  - Restlessness, thirst and air hunger
  - Circulatory failure and shock.

CLINICAL DESCRIPTION

Most young, healthy subjects can tolerate the rapid loss of 500–1000 mL of blood (10–20% of the blood volume) with few if any symptoms. About 5% of the patients, however, experience a vasovagal syncope to blood loss of this magnitude. The reaction consists of weakness, sweating, nausea, and a fall in heartrate and blood pressure, often followed by light-headedness and loss of consciousness. Whether these individuals react as they do because of physiologic or psychic differences in their makeup is unknown. However, they show two dominant personality traits: hypochondriasis and a tendency to react to stress with hopelessness, depression and despair.

With loss of 1000–1500 mL of blood (20–30% of blood volume), previously healthy subjects remain asymptomatic while at rest in a recumbent position, but they may experience light-headedness and hypotension when upright. They also respond to exertion with an unusual degree of tachycardia.

After losing 1500–2000 mL (30–40% of the blood volume), symptoms develop even when the subject is recumbent. Thirst, shortness of breath, clouding or loss of consciousness, and sweating appear. The blood pressure, cardiac output and central venous pressure

decrease. The pulse usually becomes rapid and feeble; however, the vasovagal reaction develop bradycardia.

A redistribution of blood flow occurs as the result of adrenergic stimulation; flow to the heart, lungs, liver and brain is favored at the expense of the skin, muscles, kidneys and GI tract. Consequently, the skin, especially that of the extremities, becomes cold, clammy and pale. Urine volume decreases, and salt and water are conserved because of decreased renal blood flow and the actions of aldosterone and antidiuretic hormone.

With rapid blood loss exceeding 2000–2500 mL (40–50% of the blood volume), a severe state of shock ensues. Lactate acidosis resulting from inadequate tissue perfusion may be found. At this stage, their is significant risk that the shock will become irreversible, leading to fatality.

After sufficient time has elapsed and blood volume is restored toward normal, either by physiologic mechanisms or with appropriate fluid replacement therapy. The manifestations of anemia appear now.

PATHOPHYSIOLOGY

After acute hemorrhage, physiologic mechanisms come into play to restore blood volume by an influx of fluid containing protein and electrolytes. This restoration occurs in 2 phases. The first is a partial restitution brought about in response to a fall in capillary pressure. This phase is followed by a slower restoration of both volume and protein that may take several days. This second phase results from a shift of intracellular fluid to the interstitium and from there to the intravascular compartment. In recumbent subjects, most of the expansion of plasma volume occurs within 24 hours, chiefly by movements of water and electrolytes. In ambulatory patients, the plasma volume expansion is accomplished more slowly and is related primarily to mobilisation of the extravascular albumin pool. With large hemorrhages (>30% of blood volume), the second phase is impaired by forces tending to drive fluid into cells, countering the outflow.

As restoration of volume occurs and anemia manifests, erythropoietin secretion increases. Under its influence erythropoiesis is augmented. Erythroid hyperplasia is usually perceptible 3–5 days after hemorrhage. With hemorrhagic anemia, in contrast to hemolytic anemia, the rate of red cell production may be restricted by the availability of iron, even in the absence of iron deficiency.

Iron must be drawn from ferritin and hemosiderin stored in macrophages, a relatively slow process compared to mobilization of iron newly extracted from destroyed red cells. Marrow turnover after hemorrhage may be limited to about three times the normal level, compared with five or even eight times normal in chronic hemolytic anemia states. Also iron supply may be restricted by the effects of concurrent inflammation. Surgery and trauma often trigger the mechanisms leading to the anemia of chronic disorders and the disturbance in iron metabolism that accompanies it.

MANAGEMENT

Most important is to restrict further blood loss. Simultaneously, efforts should be aimed at restoring blood volume. This procedure is more important than providing red cells. Whole blood was formerly recommended for volume expansion on the grounds that it constituted a direct replacement for what was lost. However, without losing time for typing and cross-matching one must immediately start at least normal saline or Ringer's lactate (these are less expensive and usually satisfactory). Colloids, such as albumin, dextran or hydroxyethyl starch, are more effective in restoring blood volume and can do

so with smaller infusion volumes. However, they are considerably more expensive and their clinical superiority has not been clearly established.

Once blood volume has been restored, the question of red blood cell transfusion can now be addressed. This decision rests more on a careful assessment of clinical signs and symptoms than on laboratory values. The important symptoms are syncope, dyspnea, postural hypotension, tachycardia, angina, and transient ischemic attacks. If any of these are present, transfusion of red blood cells is indicated. Administration of red cells to establish or maintain a certain blood hemoglobin level is much less important, but values below 7 g/dL normally require transfusion even in asymptomatic individuals.

Anemia following hemorrhage rarely requires specific therapy. If iron stores are adequate, the marrow is capable of regenerating the lost red cells, and the rate of regeneration is not accelerated by the administration of hematinics. As a rule, no matter how great the blood loss, the RBC count reaches 4.5 million/mm^3 by about 33 days. Hemoglobin values reach normal levels by about 2 months. Morphologic evidence of active red cell regeneration should disappear after 10–14 days, if hemorrhage does not recur. The leuocyte count should be normal after 3–4 days. Sustained reticulocytosis is suggestive of continued bleeding. Persistent leukocytosis may result from the same cause, from hemorrhage into body cavities, or from complications. The latter, particularly infections, tend to delay the hematopoietic response.

TREATMENT

- Arrest of blood loss when possible, and
- Restoration of blood volume to normal.

  On priority basis immediately give plasma expanders like dextran to maintain cardiac output. Blood transfusion may be given. As blood loss leads to iron loss, hence iron supplimentation for 1–2 months if body iron stores are inadequate.

ANEMIA OF INFECTION

- Usually occurs in chronic infections, and occasionally in acute infections.
- Besides causing anemia themselves, infections may impair the response of other anemias to treatment.

ETIOLOGY

Most common infections causing anemia are inflammation of the:

- Female pelvic organs (especially puerperal infection)
- Urinary tract infection
- Lung abscess
- Empyema
- Suppurative bronchiectasis
- Pneumonia
- Osteomyelitis
- Bacterial endocarditis
- Tuberculosis
- Typhoid
- Brucellosis
- Chronic skin ulceration with a discharging sinus
- Acute rheumatic fever.

CLINICAL ASPECTS

Clinical features are those of the causative infection predominantly, as the anemia is of moderate severity usually. However, in some cases of bacterial endocarditis, anemia may be the first presenting manifestation.

BLOOD PICTURE

- Hemoglobin generally varies from 9 to 12 g%.
- Severe anemia may occur in septicemia and massive infections, otherwise severe anemia should make one think in terms of blood loss or renal failure.
- Anemia usually takes at least 1 month to develop, progresses slowly over several months, and then tends to become stationary.
- Anemia is generally normocytic and normochromic, however, slight hypochromia and microcytosis may occur.
- The reticulocyte count and serum bilirubin are usually normal.

PATHOGENESIS

- There is mild hemolytic element.
- Bone marrow response is impaired due to:
  a. Disturbance of erythropoietin production or utilization and
  b. Disturbance of iron metabolism with impaired flow of iron from RE system to the erythroblasts.

BIOCHEMICAL CHANGES

- Serum iron decreased (average values noted = about 40 μg%)
- Serum transferrin decreased (average values noted = 200 μg%).
- Free erythrocyte protoporphyrin is increased
- Plasma copper also increases.

Blood loss and acute hemolysis are occasional contributing factors. For instance, blood loss may occur from a lung abscess or a chronic discharging sinus. The acute severe anemia which may develop with septicemia and virulent acute infections is due to both toxic depression of erythropoiesis and hemolysis. The anemia of *Clostridium welchii* is usually frankly hemolytic. Rarely, viral infections cause an autoimmune acquired hemolytic anemia. In G6PD deficiency, transient acute hemolysis may be precipitated either by the infection or by drugs given.

TREATMENT

- Eradicate the causative infection
- Iron, vitamin B_{12} and folic acid are useless
- Cobalt has been shown to correct anemia, but because of side-effects, it is not indicated
- Blood transfusion may be given prior to surgical intervention for any reason.

TUBERCULOSIS

If tuberculosis is restricted to one lung or organ, there may not be any anemia, with acute miliary spread a moderate normochromic or slightly hypochromic anemia is the rule. Rare hematological complications of tuberculosis are myelosclerosis, tuberculous splenomegaly, pancytopenia and leukemoid reaction.

CHRONIC RENAL FAILURE

Anemia is an almost invariable manifestation of chronic renal failure, often contributing substantially to the morbidity of the condition. It may be considered as typical of the disease as azotemia. Ther term anemia of chronic renal insufficiency refers to that anemia resulting directly from failure of the endocrine and excretory functions of the kidney. Kidney is the major sourse of erythropoietin, and the ability to secrete this hormone is lost as the kidney fails. In addition, renal failure is associated with retention of a variety of potentially toxic substances, including some that inhibit erythropoiesis and others that shorten RBC survival.

Lack of sufficient erythropoietin is by far the most important feature of these anemia-causing factors; consequently, the hypoproliferative features of the anemia tend to predominate. Other complicating factors also contribute. If the kidneys are infected or inflammed, the anemia of chronic disorders is likely to be observed. Iron-deficiency anemia may develop because of blood loss from the genitourinary or gastrointestinal tract or into the hemodialysis apparatus. The megaloblastic anemia of folate deficiency also may occur in patients receiving periodic dialysis, but otherwise only rarely, unless dietary intake is poor or the patient is vomiting. Certain types of renal disease, including the hemolytic-uremic syndrome and malignant hypertension, are associated with microangiopathic hemolytic anemia. Finally, aluminium intoxication can cause microcytic anemia in dialysis patients.

Anemia may sometimes be the first presentation for a patient to seek medical assistance in chronic renal failure.

ETIOLOGY

- Anemia tends to be severe in renal disease with infection, as in
  - Chronic pyelonephritis
  - Chronic nephritis
  - Cystic disease of the kidney
  - Urinary tract obstruction and
  - Renal tuberculosis
- It also occurs in systemic disease with renal involvement, as in
  - Disseminated lupus erythematosus
  - Polyarteritis
  - Amyloid diease
- Anemia is usual in malignant hypertension (in malignant hypertension and other forms of progressive renal failure, the hematological picture of microangiopathic hemolytic anemia develops due to intravascular thrombosis or fibrin deposition).

Chronic renal failure occurs during the final stages of severe renal disease. As a rule, the nature of the underlying disease bears little relation to the degree of anemia, although anemia may be less severe in patients with hypertensive renal disease and considerably less severe in patients with polycystic disease. Apparently, the erythropoietin-secreting function of the kidney is preserved in polycystic disease, even when excretory function is lost. In most cases, the patient seeks medical attention because of symptoms related to the underlying renal disease and anemia is an incidental finding. Occasionally, however, reverse may be the case. The severity of anemia bears a rough relationship to the degree of renal insufficiency. Anemia is not apparent until the creatinine clearance falls below 40 mL/minute/1.73 m^2. Between 2 and 40 mL/minute/1.73 m^2, a statistically significant correlation betwen creatinine clearance and VPRC has been observed. Similar relationship

has been reported between glomerular filtration rate (GFR) and blood hemoglobin concentration and between the blood urea nitrogen (BUN) and the degree of anemia. Oliguria is another predictor of anemia.

MECHANISMS INVOLVED IN ANEMIA OF RENAL DISEASE

Hypoproliferative Anemia

- Diminished erythropoietin—diminished erythroid committed precursors
- Suppressive effects of uraemic toxins on erythroid precursors
- Folate deficiency (hemodialysis)

Hemolytic Anemia

- Unfavorable chemical environment
- Uraemic toxins

Dilutional Anemia

Abnormal fluid retention

Blood Loss Anemia

- Gastrointestinal bleeding
- Blood drawn for laboratory tests
- Hemodialysis
- Chronic iron deficiency

Hypersplenism

Chronic renal dialysis associated splenomegaly.

BLOOD PICTURE (Fig. 7.1)

- Anemia is unusual when the blood urea is less than 7 mmol/L (40 mg%).
- Anemia is almost invariable with any significant degree of nitrogen retention. As the blood urea rises from 8 to 40 mmol/L (250 mg%) the hemoglobin progressively falls at the rate of about 2 g% for every rise of 8 mmol (50 mg%) in blood urea. A progressive severe anemia is more likely to be associated with a microangiopathic hemolytic anemia.
- The anemia is characteristically normochromic and normocytic but slight macrocytosis is usually observed. Polychromatophilia is unusual. Moderate anisocytosis is common. In later stages large numbers of 'burr' cells are seen. The appearance of schistocytes, helmet cells and microspherocytes heralds development of microangiopathic hemolytic anemia. The reticulocyte count is usually normal. TLC may be raised in association with infection. Platelet count is normal but their function is found to be abnormal in about 33% of the cases. ESR is nearly always raised.

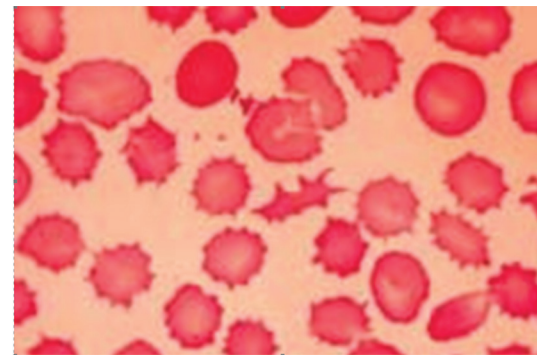


Fig. 7.1: Burr cells in renal failure, peripheral smear

OTHER LABORATORY FINDINGS

Anemia may progress as renal failure worsens but in most cases VPRC usually stabilizes between 15 and 30%. Because regulation of body water and electrolyte balance is impaired in renal disease, both hydremia and dehydration are common. Consequently, the apparent degree of anemia may be exaggerated or minimised by alterations in plasma volume.

At very high BUN values, reticulocytosis may reach 6%. TLC is often normal, but sometimes slight neutrophilic leukocytosis may be observed. The bone marrow tends to be moderately hypercellular and slight erythroid hyperplasia may be observed, average M:E ratio being 2.5:1. When renal failure is relatively acute, hypoplasia of erythroid elements is found. Values for serum iron vary considerably in renal disease. As a rule, the value is normal when renal impairment is mild. The amount of free erythrocyte protoporphyrin (also called erythrocyte zinc protoporphyrin, EZP) may be normal or moderately increased. The erythrocyte LDH is within normal limits. The hemoglobin A1 fraction tends to be higher in uremic patients. The magnitude of the increase usually correlates with the average value of BUN over the preceding 2–3 months.

The increase is thought to result from carbamylation of the hemoglobin molecule by urea-derived cyanate; it can be detected by column chromatography but not by using certain chemical methods designed for detecting glycosylated hemoglobin in diabetic patients. The increase in A_1 fraction may continue even after successful renal transplantation has brought the azotemia under control; under such circumstances, the increase might reflect disturbed carbohydrate metabolism.

PATHOGENESIS

Three factors are involved in the pathogenesis of anemia associated with chronic renal failure

1. Most important being impaired secretion of erythropoietin
2. Marrow depressive effect of retained toxic substances
3. Shortened erythrocyte life.

As excretory function of the kidney decreases, so does its ability to secrete erythropoietin. Radioimmunoassay studies have shown an increase in erythropoietin levels. Immunologically active, this erythropoietin is biologically inactive. Furthermore, the usual negative correlation between serum levels of erythropoietin and hemoglobin is lost. Extrarenal sources of erythropoietin exist in our body but production by these sources cannot offset the anemia as is possible by the renal origin erythropoietin.

Retained uremic toxins may depress erythropoiesis. Erythropoiesis improves in dialysed patients. Uremic plasma can depress heme synthesis and/or inhibit growth of erythroid colonies *in vitro*. Secondary hyperparathyroidism may also contribute to marrow depression in patients with renal failure. The VPRC increases modestly in about 40–50% of patients with renal failure who undergo parathyroidectomy.

Administration of erythropoietin relieves anemia to an extent. The occasional failure to respond to erythropoietin generally can be attributed to the coexisting iron deficiency or other complications.

A third pathogenetic factor is hemolysis. Erythrocyte survival often within normal limits, may be slightly to moderately reduced. Red cell life reduces as azotemia increases.

DIAGNOSIS

Chronic renal failure should be considered as a possible cause for any normochromic anemia in which the etiology is not obvious; seek a detailed history, especially pertaining to urinary system.

TREATMENT

Anemia is refractory to all measures except those that reduce blood urea. The availability of recombinant human erythropoietin has made considerable difference in the well-being of these patients.

Recombinant human erythropoietin (rHuEPO) has been used for treatment of the anemia of renal disease. It is safe and effective and it substantially improves the quality of life and cognitive functions. Although costly, it is often the treatment of choice. It can be given intravenously or subcutaneously. Iron supplementation is also necessary. Lesser therapeutic roles are ascribed to folate administration, blood transfusions and other rarer forms of therapy.

Early Treatment

In some patients, anemia develops before specific treatment of the renal failure is necessary. rHuEPO helps with good results. 50 IU/kg subcutaneously three times weekly, suffices, physical and intellectual abilities improve.

Homotransplantation

In many ways, renal transplantation is the most complete and satisfactory treatment for renal insufficiency. When a renal homograft is successful, both the endocrinologic and excretory functions of the kidney are restored. With a successful graft, the hematologic response is gratifying. Anemia is usually corrected in 2–3 months, this occurs on account of erythropoietin secretion by the grafted kidney. About 20% of these patients do not behave in this way. Failure to respond usually can be explained on the basis of hemorrhage, vigorous immunosuppression or graft rejection.

Hemodialysis

About 60% of the patients (more so in India) with end stage renal disease are treated with maintenance hemodialysis. It controls most of the symptoms and biochemical manifestation of renal failure, but erythropoietin secretion does not improve and anemia continues to be a major source of morbidity. PCV usually stabilizes at 15%. After 1–10 months or more of treatment, modest improvement in degree of anemia or in transfusion requirements has been observed. The improvement results chiefly from an increase in red cell production, even though erythropoietin levels do not increase. The use of rHuEPO has revolutionized the care of hemodialysis patients. It is a safe but an expensive mode of treatment. Initial dose is 50–100 IU/kg three times weekly, given IV or SC. Further dose is adjusted depending upon the response. An additional increment of 12.5–50 IU/kg is added to each dose. Once the target range of 9–11 g/dL of hemoglobin is achieved, the maintenance dose required is often less than 150 IU/kg. Over 97% of cases respond and PCV rises from 22–35% within 3 months. The need for erythrocyte transfusion is eliminated in all patients within 2 months.

Other treatments include blood transfusions and androgens, but their use is rendered obsolete with the advent of rHuEPO. In some patients where splenic sequestration of erythrocytes occurs, splenectomy is known to help.

ERYTHROPOIETIN RESISTANCE

Causes of resistance to erythropoietin therapy

1. Iron deficiency
2. Insufficient dialysis
3. Aluminium intoxication
4. Hyperparathyroidism
5. Vitamin B_{12} or folate deficiency
6. Blood loss
7. Inhibitor cytokines
   - Infections
   - Inflammation
   - Neoplasms
8. Angiotensin-converting enzyme (ACE) inhibitors
9. After failed homograft
10. Hemoglobinopathies
11. Unknown factors.

Loss of iron occurs with repeated dialysis. Patients with serum ferritin levels below 100–200 µg/L or with transferrin saturation values less then 20–25%, require iron supplementation. Some therapists recommend iron supplementation for all patients. Iron can be given IV if compliance is poor to oral iron administation.

Secondary hyperparathyroidism often accompanies renal failure, and the associated marrow fibrosis may contribute to the anemia and to erythropoietin resistance. Treatment with vitamin D_3 can decrease rHuEPO requirements and improve hemoglobin values. Many patients require vitamin B_{12} and folate administration as well.

Side effects of rHuEPO: No important side effects have been associated with rHuEPO administration in diseases other than renal failure. When it is used for anemia in renal disease, however, increased blood pressure is an important complication (seen in up to 35% of cases). Occasionally, the hypertension is abrupt and severe with encephalopathy and seizures. The pathogenesis of hypertension is incompletely understood and probably multifactorial.

Peritoneal Dialysis

Fair number of end-stage renal disease patients are treated with peritoneal dialysis and the severity of anemia is less marked in these patients as compared to those on hemodialysis peritoneal dialysed patients can also be given rHuEPO. Administration of 20 IU/kg three times weekly or 10 IU/kg daily by SC route helps a great deal.

MALIGNANCY

- Anemia is a usual accompaniment of malignancy.
- Factors responsible for causing anemia in a patient with malignant disease.

Common

- Blood loss
- Infection
- Bone marrow metastases.

Less Common

- Disturbance of nutrition
- Impaired renal function

- Anemia of malignancy (like anemia of chronic infection)
- Hemolytic anemia
- Sideroblastic anemia
- Bone marrow depression from treatment of malignant disease.

MECHANISMS OF ANEMIA IN MALIGNANCY

Direct Effects

Replacement of marrow by malignant cell

Primary Hematologic Malignancy

- Ineffective erythroid production
- Qualitative reduction in eyrthropoiesis

Metastatic Marrow Infiltration

- Quantitative reduction in erythropoiesis
- Replacement of marrow by fibrosis

Indirect Effects

- Anemia of malignant disease
- Anemia of associated organ failure (e.g. renal, hepatic)
- Malnutrition and vitamin deficiency
- Microangiopathic hemolytic anemia
- Immune hemolytic anemia

Treatment Associated Anemia

Immediate

a. Chemotherapy
b. Radiation therapy

Late

a. Secondary myelodysplasia/leukemia
b. Idiopathic
   - ?depleted marrow reserve
   - Microanglopathic hemolytic anemia (post-mitomycin)

TYPE OF ANEMIA

The anemia is usually normocytic but hypochromic microcytic anemia of iron deficiency occurs in patients with chronic blood loss. Occasionally a slight to moderate macrocytosis is present, e.g. with the leukoerythroblastic anemia of secondary bone metastases, or when reticulocytes are increased either as a result of hemorrhage or hemolysis.

Other blood changes include leukocytosis because of a necrotic and infected tumor, raised ESR, changes in iron metabolism, coagulation disorders (DIC). Eosinophilia is also relatively common.

ANEMIA ASSOCIATED WITH SECONDARY CARCINOMA OF THE BONE MARROW

After lungs, liver, the bone marrow is the third most important site for blood-borne metastases. Breast and prostate (quite commonly) and carcinomas of lung, thyroid, kidney, stomach and malignant melanomas also commonly metastasise to bone marrow. Metastatic growths most frequently occur in the site normally occupied by the red bone marrow namely, the vertebrae, ribs, sternum, pelvis, skull and upper ends of the femora and humeri. When secondary growths occur in yellow-marrow sites, they evoke red marrow transformation and are ringed by it. Extent of bone marrow involvement does not necessarily correspond to degree of anemia evoked.

BLOOD PICTURE

The blood picture is usually a normochromic normocytic anemia or pancytopenia or that of a leukoerythroblastic anemia, i.e. characterized by the presence of immature red and white cells. Anemia is often only moderate but with progression of disease it becomes severe.

Slight hypochromia may be present; moderate to marked anisopoikilocytosis are common. Macrocytes are often present in moderate numbers. Moderate polychromasia and basophilic stippling are common. Reticulocyte count may be raised. Normoblastemia may be 10 normoblasts per 100 WBCs (normoblasts are usually late and intermediate but sometimes early normoblasts can also be seen). This picture brings in the differential diagnosis of microangiopathic hemolysis, often associated with mucin secreting adenocarcinomas and usually associated with red cell fragmentation, thrombocytopenia and sometimes with a coagulation disturbance. Megakaryocytes may be rarely seen and be confused with circulating tumor cells.

The TLC is variable, the DLC shows a shift to left (irrespective of the total count). Moderate to marked thrombocytopenia is usual. ESR is usually raised. Occasionally, the red cell osmotic fragility is raised and rarely the Coombs' test is positive.

BONE MARROW

Tumor cells may be seen in the aspirated marrow but trephine biopsy yields better results. Aspiration may sometimes yield a dry or blood tap. A dry tap may result if the site of puncture has been replaced by fibrous tissue or bony tissue. A negative biopsy does not necessarily rule out marrow infiltration. Tumor cells are characteristically large cells which appear in clumps. It is sometimes necessary to examine six or more films before tumour cells can be seen.

CLINICAL ASPECTS

The clinical features are those of:

1. The anemia (weakness, lassitude, pallor, dyspnea, etc.)
2. The manifestations of bone involvement (fractures, spontaneous bleeding)
3. The primary tumor (weight loss, fever), and
4. Metastatic involvement of other organs (hepato- and/or splenomegaly, jaundice).

DIAGNOSIS

When primary site is obvious and radiological changes are present, the diagnosis of metastatic bone carcinoma is simple. The raised serum alkaline phosphatase provides ample evidence.

Diagnostic problem may arise when (i) anemia or bleeding is the presenting manifestation, and (ii) when radiological bony changes are absent.

PROGNOSIS

Leukoerythroblastic anemia occurs late in the disease and is a poor prognostic sign. In untreated patients death usually occurs in less than a year, often within 1 or 2 months.

LIVER DISEASE

Anemia is a common feature of liver disease, especially with cirrhosis (occurs in 66% of cirrhotics).

Anemia is particularly characteristic of Laennec cirrhosis. Changes in the morphologic characteristics of red cells, reduced red cell survival, and even mild anemia, however, have been reported in association with certain other liver diseases, including biliary cirrhosis, hemochromatosis, postnecrotic cirrhosis, and acute hepatitis. Anemia is also associated with Wilson's disease.

Many complicating factors contribute to the development of anemia in patients with alcoholic liver disease. Of particular importance is suppression of bone marrow by alcohol. Chronic alcoholics can develop a characteristic sideroblastic anemia, often accompanied by impaired folate metabolism or overt folate deficiency. Also common in cirrhosis is hemorrhage especially from the upper gastrointestinal tract, but loss of blood from the nose, hemorrhoids, and the uterus often occurs in association with a common disturbance of hemostasis. Mild-to-moderate anemia may be associated with liver disease in the absence of the aforementioned complicating factors. It is referred to as the "uncomplicated" anemia of liver disease. To a greater extent–hypervolemia, shortened red cell survival, and impaired ability of the marrow to respond optimally to anemia—are held responsible for this.

About 75% of patients with chronic liver disease develop anemia as defined by VPRC or hemoglobin. The whole blood volume averages about 15% higher than normal and this hemodilution tends to exaggerate the prevalence and degree of anemia. The magnitude of hypervolemia in Laennec's cirrhosis appears to be related to the degree of portal hypertension, but not to the presence or absence of ascites.

The anemia is usually mild to moderate. Hemoglobin rarely falls below 9 g/dL in the absence of complications. About 5% of cases of relatively severe hepatocellular disease develop spur cell hemolytic anemia and hemoglobin may reach up to 5 g/dL. Spur cell anemia may be chronic accompaniment of alcoholic cirrhosis, or it may develop as liver function deteriorates within weeks to months of death. If liver function improves, remission may occur. Spur cell anemia also affects infants with cholestatic liver disease. Episodes of hemolytic anemia may also develop in alcohol abusers with relatively mild liver disease. These episodes, mild to moderate in severity, tend to be self-limited and resolve in 2–4 weeks if alcohol is withdrawn. If accompanied by jaundice and hyperlipidemia, the condition sometimes known as Zieve's syndrome; however, hyperbilirubinemia and hyperlipidemia are not implicated in the hemolysis. Liver at this stage shows fatty infiltration.

MECHANISMS INVOLVED IN ANEMIA OF LIVER DISEASE

1. Defective erythrocyte development
   a. Ineffectie erythropoiesis
      - Folate deficiency (poor nutrition, alcoholism)
      - Iron deficiency (associated GI bleeding)
      - Pyridoxine deficiency (alcoholism)
      - Protein deficiency (hypoproteinemia)

b. Hypoproliferative anemia
   - Anemia of chronic disease (e.g. chronic hepatitis)
   - Primary viral suppression (hepatitis B, EBV, CMV)
   - Toxic suppression (impaired liver clearance)
   - Alcoholic bone marrow suppression.

2. Dilutional anemia
   - Elevated plasma volume in cirrhosis
3. Hypersplenism
   - Erythrocyte sequestration with congestive splenomegaly
4. Hemolytic anemia
   - Spur cell anemia (abnormal membrane lipids)
   - Acanthocytosis
5. Blood loss anemia
   - Bleeding from esophageal varices
   - Bleeding associated with coagulopathy of liver disease.

The blood picture varies with the causative factors. Anemia does not parallel the severity of altered liver function tests. Hemoglobin is usually about 9 g%. Lower values should arouse suspicion of some complicating factor, e.g. blood loss or nutritional megalobastic anemia. The anemia is macrocytic or normocytic and normochromic. Three types of macrocytes are described:

Thin Macrocyte

Thin macrocyte occurs in response to nonspecific liver damage, bone marrow shows macronor-moblastic reaction, it may occur in all types of liver damage and obstructive jaundice.

Thick Macrocyte

Thick macrocyte (MCV increased) occurs only in patients with hepatic disease with protein malnutrition, bone marrow shows atypical megaloblastosis.

Target Macrocyte

Target macrocyte target cells are commonly present in moderate numbers especially in jaundiced patients.

Moderate reticulocytosis (up to 5%), together with moderate polychromasia and basophilic stippling is seen.

When spur cell anemia supervenes, characteristic acanthocytes – erythrocytes covered with 5–10 spikelike projections are evident. The acanthocytes are morphologically indistinguishable from the distorted erythrocytes found in patients with abetalipoproteinemia. Stomatocytes are sometimes observed in association with the transient hemolytic episodes associated with acute fatty liver disease, but they are noted in alcoholics who display no evidence of hemolysis.

About 50% of patients with cirrhosis have mild thrombocytopenia (averaging above 50,000/mm^3), platelet function too may be impaired.

TLC is normal in the absence of complications. Leukopenia may result from hypersplenism or megaloblastic anemia and leukocytosis from acute bleeding infection, etc. There is often a mild absolute lymphopenia irrespective of TLC, ESR is often raised.

Bone marrow cellularity varies, reaction is normo- or macronormoblastic. Leukopoiesis is normal as are the megakaryocytes. Plasma cells may be increased. The iron content is normal.

ERYTHROKINETICS AND PATHOGENESIS

Red cell survival tends to be moderately shortened in patients with alcoholic liver disease. The rate of red cell destruction or disappearance correlates well with anemia. Erythrocyte survival is particularly likely to be reduced in patients with predominant indirect bilirubinemia, that is, when the indirect fraction is greater than 2.5 mg/dL and constitutes more than 70% of the total. Erythrocyte survival is significantly shortened in patients with biliary cirrhosis, obstructive jaundice, and infectious hepatitis, even in the absence of anemia. Hemolytic factor when present in chronic liver disease is thought to be of extracorpuscular origin. Congestive splenomegaly with augmented splenic sequestration as well as excessive splenic conditioning and destruction of red cells, is probably the most important extracorpuscular factor. Sometimes, splenectomy may help a few patients. Abnormal erythrocyte metabolism is another possible factor leading to reduced erythrocyte survival in liver disease. The possible reasons are NADP deficiency, hypophosphatemia with reduced erythrocyte ATP levels and consequent hemolysis.

Characteristic red cell membrane lipids are found in patients with hepatits, cirrhosis, and obstructive jaundice. In the usual uncomplicated case, a 25–50% increase in both cholesterol and lecithin is noted in the membrane. These changes result in an increased cell surface area associated with the target cells or thin microcytes that are so characteristic of liver disease. No good evidence exists that such abnormalities shorten the erythrocyte life span.

The loss of sialic acid from the red cell surface may contribute to impaired viability of the cell. Bile duct obstruction leads to an increase in the activity of neuraminadases, enzymes that remove sialic acid.

In spur cell hemolytic anemia, the erythrocyte membrane accumulates excess cholesterol, without a corresponding increase in lecithin. This change is accompanied by pronounced reduction in erythrocyte survival, probably because the distorted cells are less deformable than normal and thus become trapped by splenic macrophages. Splenic conditioning aggravates the abnormality by causing loss of cell surface with transformation of echinocytes to acanthocytes. Splenectomy may bring about reduction in hemolysis, but an intact spleen is not required for the development of spur cell anemia. The mechanism whereby the red cell lipid pattern becomes altered is ill understood. One possible factor is an alteration in the cholesterol-phospholipid ratio in high density plasma lipoproteins. Mature erythrocytes are unable to synthesize lipids and depend largely on plasma lipoproteins for renewal and replacement. In addition, red cells normally are actively involved in phospholipid repair, and the repair process appears to be defective in spur cell anemia. Phospholipid biosynthesis is inhibited and therefore cannot compensate for the increased membrane cholesterol characteristic of liver disease. Other possible factors in the development of altered membrane lipids are changes in plasma lecithin-cholesterol acyltransferase (LCAT) activity, retention of lytic bile acids, and an increase in the intrinsic proteolytic activity of the membrane.

TREATMENT

The anemia in liver disease responds only to improvement in the liver function.

COLLAGEN DISEASE

RHEUMATOID ARTHRITIS

Anemia in these patients is usually mild to moderate. Two types of anemias may be seen.

1. The normocytic normochromic one (more common) that parallels the severity of disease, and
2. Microcytic hypochromic anemia, due to concomitant iron deficiency as a result of blood loss arising because of chronic aspirin ingestion.

Leukocytosis may occur in acute phases. Eosinophilia occurs occasionally. ESR is raised. The reticulocyte count and serum bilirubin are normal. Bone marrow is essentially normal.

Pathogenesis

The mechanism of anemia of rheumatoid arthritis is more or less similar to that of the anemia of chronic infection. Treatment is to combat the disease itself, remission of the disease is spontaneously followed by a rise in the hemoglobin. Iron may be given in cases showing microcytic hypochromic anemia.

SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

Hematological changes almost invariably occur at some stage of the disorder. Occasionally, ITP and acquired hemolytic anemia may be the first manifestations.

Anemia

Anemia occurs in about 75% of the cases. It is usually normocytic normochromic or slightly hypochromic and of mild to moderate severity, lower values may occur in patients with acquired hemolytic anemia or renal insufficiency. Autoimmune acquired hemolytic anemia with a positive Coombs' test develops in about 5% of the cases.

White Cell Count

It is often low (may be variable). The DLC shows greater reduction in lymphocytes than neutrophils.

Thrombocytopenia

Moderate symptomless thrombocytopenia is common. The ESR is raised in most of the patients. Values over 100 mm/hour (Westergren's) are not uncommon. A false-positive Wassermann reaction occurs in about 20% of cases and the serum complement is lowered in the presence of active renal involvement. A raised serum globulin is common and antibodies to DNA are usually detectable by a number of methods. Bone marrow is normal except for a moderate increase in plasma cells.

LE Cell Phenomenon

LE cell was first described in 1948 in bone marrow of the DLE patients. It can be shown in peripheral blood also. It is positive in about 82% of established SLE cases. A positive test is pathognomic of lupus, especially in patients with typical clinical features. Positive LE cells can also be found in other collagen disorder, chronic lupoid hepatitis, drug induced lupus syndromes (especially with procainamide) (Fig. 7.2).

The LE cell consists of a leukocyte, almost invariably a neutrophil, whose cytoplasm contains a large, spherical, opaque, structureless homogenous body, which stains pale purple with Romanowsky stains. The nucleus of the cell is usually displaced to one side and may appear to be wrapped around the ingested material. The LE cell must be distinguished from the "Tart cell", which is often found in LE cell preparations and may cause confusion. The 'Tart cell' is a monocyte or neutrophil which has phagocytosed another cell or the

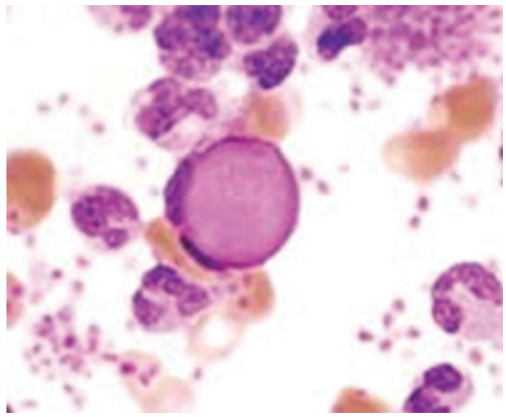


Fig. 7.2: LE cell

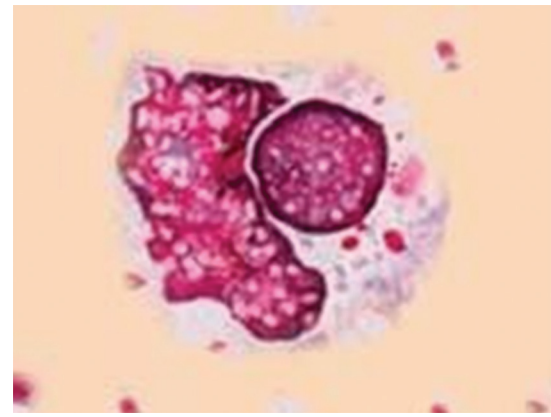


Fig. 7.3: Tart cell

nucleus of another cell. It differs from LE cell in that the phagocytosed nuclear material either has a definite nuclear structure or is pyknotic and stains much more deeply than an LE body. The significance of 'Tart' cell is as yet not known (Fig. 7.3).

The LE cell phenomenon depends on the presence in plasma of an immunologically distinct gamma globulin, the LE factor, which has the characteristics of an antibody of the IgG type. This factor has the property of causing *in vitro* lysis of the nuclei of neutrophils; as it reacts with autologous nuclei it can be regarded as an autoantibody. The neutrophil whose nucleus has undergone lysis ruptures liberating the lysed nuclear mass, which is then phagocytosed by other neutrophils. The exact mechanism for this is ill-understood. It is thought that the LE factor is an autoantibody to deoxyribonucleohistone, and that the formation of the LE cell is initiated by its actions on the nucleoprotein nucleus of the cell. Antibodies to other nuclear consitutents may be present but they do not appear to be capable of initiating LE cell formation; however, they may contribute to the final morphological appearance of the altered nucleus.

ENDOCRINE DISORDERS

ANEMIA ASSOCIATED WITH ENDOCRINE DISORDERS

Anemia commonly accompanies disorders affecting the thyroid gland, adrenal glands, parathyroid glands, gonads, or pituitary gland. Generally, the anemia produced in mild to moderate and produces no symptoms. In fact, the amount of circulating hemaoglobin may be entirely appropriate to the needs of the organism, because the metabolic disturbance often results in reduced oxygen requirements. In most patients, these endocrine disorders begin insidiously. The early symptoms are nonspecific and include fatigue. Consequently, when the blood hemoglobin level is subnormal, the symptoms may be ascribed to the anemia, and the diagnostic efforts may be directed toward the hematopoietic system. Few morphologic abnormalities are observed, and none is specific. Unless the clinician suspects and searches for endocrine disease, the diagnosis may be overlooked.

MYXEDEMA

Anemia has been observed in 20–60% of patients with hypothyroidism. Although hypothyroidism is more common in women than in men, anemia is more common in

hypothyroid men than hypothyroid women. Three morphologic types of anemia have been described in adult patients.

a. When hypochromic, microcytic anemia is found in association with myxedema, it almost always is attributable to iron deficiency. Serum iron values are subnormal and the anemia responds to iron therapy, even if thyroid hormone is not administered; it is not relieved by hormone treatment if iron is withheld. Often iron deficiency in myxedema produces normocytic anemia. Iron deficiency occurs in association with myxedema in part because menorrhagia is common manifestation of the illness, in part because the achlorhydria found in myxedema subjects may lead to impaired absorption of food iron, and in part because thyroid hormone itself may be essential for normal iron absorption.
b. Distinctly, macrocytic anemia usually results from complicating deficiency of vitamin B_{12} or folate. The increased incidence of pernicious anemia in thyroid disease possibly is the result of an autoimmune mechanism. Folate deficiency may be dietrelated.
c. If folate, vitamin B_{12} and iron deficiencies are eliminated, a significant population of hypothyroid patients with anemia remain called uncomplicated anemia of hypothyroidism, and in a manifestation of the hormone deficiency itself. All or nearly all children with anemia and hypothyroidism have the uncomplicated form of the disease. The anemia is usually mild with PCV rarely falling 35%. The degree of anemia is related to both the severity and the duration of the hypothyroidism the PCV (VPRC) continues to fall for as long as 6 months after thyroidectomy in previously euthroid individuals, even though the basal metabolic rate remains at a stable, reduced level.

The MCV may be increased in hypothyroid patients, even in the absence of anemia. Anisocytosis, poikilocytosis, or other red cell morphologic abnormalities are not impressive, but acanthocytes are apparent in about 20% of patients. Usually the leukocyte and platelet counts are within the normal range, although both may be slightly reduced. The bone marrow may be mildly hypoplastic, but the M:E ratio is not significantly altered. Hemoglobin A_2 levels are mildly diminished.

The response of the anemia of hypothyroidism to thyroid hormone is sluggish. Characteristically no distinct reticulocytosis occurs and the VPRC (PCV) returns to a normal value only gradually over a 6-months period. The MCV alsmot invariably falls, even if anemia is not present or if the MCV is in the normal range when therapy is instituted. The MCV tends to reach stable levels after about 4 months or more.

HYPERTHYROIDISM

About 10–25% of these patients suffer from an unexplained mild anemia. In patients who are anemic, the hyperthyroidism tends to be of unusual severity or prolonged duration. In a larger number of patients the hemoglobin value falls a little but remains within normal limits. MCV is either normal or modestly decreased, even though iron metabolism is not disturbed (MCV may be decreased in non-anemic patients of hyperthyroidism). HbA_2 levels are slightly increased but not to the same degree as noted in patients with β-thalassemia minor. Both the anemia and the microcytosis are corrected when the hyperthyroidism is successfully treated; the pathogenesis of the anemia and microcytosis is not well understood.

HYPOGONADISM

Post-puberty, values for VPRC, blood hemoglobin concentration, and red cell count average about 10–13% higher in men than in women. In eunuchoid or castrated men,

these values fall to within the normal female range. Urinary erythropoietin levels in normal men are about three times than found in women.

The differences between the sexes are accounted for chiefly by the stimulating effect of androgens on erythropoiesis. The administration of androgens to castrated males restores male values for hemoglobin concentration. Androgens act by increasing renal synthesis of erythropoietin. Estrogens are supposed to exert a suppressive effect, possibly by suppressing hepatic synthesis of erythropoietin.

HYPOPITUITARISM

Moderately severe, non-progressive anemia is a well-recognized feature of pituitary insufficiency, regardless of cause. Average values being 10 g/dL in previously healthy subjects. Anemia is also evident in prepubertal dwarfs; however, the degree of anemia may be obscured by contraction of the plasma volume. The anemia is usually normocytic, normochromic, and the red cells are morphologically normal. Results of kinetic studies demonstrate reduced red cell production. The anemia of hypopituitarism results chiefly from deficiences of the hormones of target glands controlled by the pituitary, especially the thyroid and adrenal hormones, but also androgens. In addition, lack of other pituitary factors, such as growth hormone, prolactin, or other poorly characterized pituitary principles, may be of importance. In experimental animals, hypophysectomy results in moderately severe, slightly hypochromic and microcytic anemia associated with a pronounced decrease in erythroid elements in the bone marow. No hemolysis occurs; in fact, the red cell survival is prolonged.

As suggested for the anemia of hypothyroidism, panhypopituitarism probably produces its effects on erythropoiesis chiefly by reducing tissue oxygen consumption. The organism reacts to this decreased need for oxygen by secreting less erythropoietin, and the red cell mass diminishes until a new equilibrium between oxygen supply and demand is established. Treatment with a combination of thyroxine, cortisone and growth hormone corrects both the anemia and the marrow hypoplasia, and is more effective than any single hormone given by itself.

HYPERPARATHYROIDISM

Anemia is a rare complication of primary hyperparathyroidism. Anemia is normocytic normochromic without reticulocytosis. Leukopenia or thrombocytopenia do not occur. The cause for anemia in these patients remains obscure. Although renal failure and gastrointestinal bleeding occur in association with hyperparathyroidism. It is also thought parathormone in excess hampers the growth of erythroid precursors, and additionally it also caused marrow fibrosis. When hyperparathyroidism is secondary to renal disease; it is difficult to ascertain the relative importance of the hormone excess versus the erythropoietin deficit characteristic of renal failure.

ADDISON'S DISEASE

The total red cell volume is slightly diminished and a mild normochromic normocytic anemia is common. However, the decrease in plasma volume associated with untreated Addison's disease results in hemoconcentration and thus tends to mask the anemia. Correction of hemoconcentration by adequate therapy is often followed by an initial fall in hemoglobin and hematocrit values, serial determination of which assist in evaluating the effect of therapy. The TLC and neutrophil counts are normal. Occurrence of infections is not accompanied by neutrophil leukocytosis; this is of practical importance as a crisis

is often precipitated by infection, the presence of which cannot be ruled out by a normal white cell count.

PROTEIN MALNUTRITION

The protein globin, which constitutes 96% of the hemoglobin molecule, contains a number of essential amino acids, i. e. amino acids which the body itself cannot synthesize. Therefore, adequate dietary supply of proteins is essential for hemoglobin and other body proteins production. Hemoglobin has a high priority for available protein and it has been shown that in states of protein deficiency hemoglobin formation takes precedence over serum protein and tissue protein breakdown. In man, therefore, because of high priority of hemoglobin for the available protein, protein deficiency alone does not often act as a limiting factor in hemoglobin synthesis and cause anemia.

Anemia due to protein deficiency is seen most commonly in pregnant women in association with kwashiorkor. Pure protein deficiency would lead to a mild-to-moderate normocytic anemia. In the clinical situations where protein malnutrition exists, there are often associated deficiencies especially of iron and folate. So really, the cell size may vary as would the hypochromia. Supplementation and dietary improvement would help combat the condition.

SCURVY

Anemia is usual but not invariable in vitamin C deficiency. In adults it is normocytic normochromic but in infants and children it is often microcytic hypochromic because of associated iron deficiency. The anemia of scurvy is typically moderate in degree and it parallels the degree of deficiency. The anemia appears to be primarily due to impairment of erythropoiesis, but hemolytic element is also said to be present. TLC, DLC and platelet counts are usually normal.

PHYSIOLOGICAL ANEMIA OF PREGNANCY

The anemia in pregnancy commences about the eighth week, progresses steadily until the thirtysecond week, following which there is a progressive rise until term. There is individual variation in the degree of hemoglobin fall. In India less than 10 g% is considered significant. The PCV too, proportionately, falls with hemoglobin. Following delivery, hemoglobin and PCV values increase and return to normal by about the third month of puerperium.

The red cells are normocytic but mild anisocytosis is not uncommon with an increased percentage of slightly microcytic cells. On the stained peripheral smear the red cells appear to be normochromic, although the MCHC is often at the lower limit of normal.

PATHOGENESIS

Physiological anemia of pregnancy has been attributed to hemodilution which occurs in pregnancy. The blood volume rises during pregnancy, due to an increase in both the total red cell and the plasma volume. But plasma volume rise is by about 35–40% whereas RBC volumes rise only by about 17%. This disproportionate increase in plasma volume results in hemodilution and thus in a fall in hemoglobin value.

In addition, the demands of the growing fetus place the expectant mother at greater risk for nutritional anemias, especially deficiencies of iron and folate. Pregnant patients

are, of course, also susceptible to the same diseases that afflict other women. A hemolytic anemia may be detected in preeclamptic and eclamptic patients. The syndrome is called HELLP (for hemolysis, elevated liver enzymes and low platelets).

In addition it has been found that in most patients the fall in hemoglobin can be wholly or largely prevented by the routine administration of iron. Depletion of bone marrow iron stores is common in pregnancy. Thus, it appears that the physiological anemia of pregnancy is due to a combination of hydremia and relative iron deficiency. Treatment is iron supplementation (preparations with minimal gastrointestinal irritative effects should be given). Should complications arise, pre- or postpartum, they should be adequatly treated.

NUTRITIONAL ANEMIA OF PREGNANCY

Iron deficiency is a common complication of pregnancy and the most common cause of true anemia. This is more common in the lower socioeconomic group. Limited iron reserves even before pregnancy are common, and the additional iron requirements of pregnancy impose a negative iron balance unless supplementation is provided. Pregnant women may experience certain manifestations of iron deficiency in addition to those typical of iron deficiency anemia in other. There is evidence that the risks of prematurity, low birth weight babies, and even infant death are inceased in severe iron deficiency. The placenta becomes hypertrophied, a possible cause for maternal hypertension. A special type of pica, namely, ingestion of baking powder, can lead to symptoms suggesting eclampsia dietary supplementation is essential.

Macrocytic anemia of pregnancy is often megaloblastic and in most cases result from deficiency of folic acid. When it occurs, it often begins in the third trimester, or shortly after delivery. Folate requirements increase during pregnancy, and the diets of many pregnant patients are insufficient to meet the increased need. Folate deficiency occurs regardless of economic status of the patient, especially if they are adolescents. In uncomplicated pregnancies the gastrointestinal absorption of dietary folate (polyglutamate) and of folic acid (monoglutamate) is normal. Folate deficiency is clearly associated with fetal neural tube defects and cleft palate, but these defects are established very early in fetal development, long before frank megaloblastic anemia is detected. Women must take folate supplements even before pregnancy can be accurately diagnosed if these complications are to be avoided. Prematurity may also be a consequence of folate deficiency. Other complications of pregnancy, such as an increased incidence of abruptio placentae, uterine bleeding and reduced birth weight have also been attributed to folate deficiency by some specialists. A positive association has been noted, between bacteriuria and folate deficiency, in pregnant patients. Very rarely, the folate deficient pregnant patient may have erythroblastopenia and bone-marrow changes so closely stimulate acute leukemia, because of the predmonince of granulocyte precursors, that the best of hematologists may also diagnose it as such. Such a misinterpretation should be avoided and folate be administered with a repeat study of bone marrow to observe changes which will revert towards normalcy in a folate deficient subject.

Diagnosis depends on, first, identifying megaloblastic anemia. An increased MCV with oval macrocytes and hypersegmented granulocytes on peripheral smear may be adequate for this purpose but bone marrow examination can also be done to confirm. Next folate deficiency must be distinguished from vitamin B_{12} deficiency. The latter is rare in pregnancy, and the distinction can be made on clinical gounds, especially on the basis of a careful nutritional history. If in doubt, folate and vitamin B_{12} levels can be conducted.

Dietary supplementation with about 0.3 mg of folic acid daily during pregnancy reduces the occurrence megaloblastic anemia significantly. A supplement of 0.45 mg daily result in supranormal values for serum folate, but, even with this amount of supplementation, megaloblastic anemia may occur in a pregnant patient in whom the course is complicated by urinary tract infection or by hemorrhage. A supplementation by 0.4 mg of folic acid is supposed to be enough, wherever necessary, the same can be increased to 1 mg/day. Vitamin B_{12} deficiency in pregnant pernicious anemia patients has to be tackled by giving vitamin B_{12} injections accordingly.

ANEMIA OF CHRONIC DISORDERS

The mild-to-moderate anemia that is often found in patients with infections, inflammatory, or neoplastic diseases that persist for more than 1 or 2 months is called the anemia of chronic disorders, or the anemia of chronic disease. The characteristic feature of this syndrome is the occurrence of hypoferremia in the presence of ample reticuloendothelial iron stores. As so defined, the syndrome does not include anemia caused by marrow replacement, blood loss, hemolysis, renal insufficiency, hepatic disease, or endocrinopathy, even when these disorders are present. The anemia of chronic disorders is extremely common, and overall is probably more common than any anemia syndrome other than blood loss with consequent iron deficieny.

CONDITIONS ASSOCIATED WITH ANEMIA OF CHRONIC DISORDERS

Chronic infections

- Pulmonary infections: Abscesses, emphysema, tuberculosis, pneumonia
- Subacute bacterial endocarditis
- Pelvic inflammatory disease
- Osteomyelitis
- Chronic urinary tract infection
- Chronic fungal disease
- Meningitis
- HIV

Chronic, noninfectious inflammations

- Rheumatoid arthritis
- Rheumatic fever
- Systemic lupus erythematosus
- Severe trauma
- Thermal injury
- Sterile abscesses
- Vasculitis

Malignant diseases

- Carcinoma
- Hodgkin's disease
- Lymphosarcoma
- Leukemia
- Multiple myeloma

Miscellaneous

- Alcoholic liver disease

- Congestive heart failure
- Thrombophlebitis
- Ischemic heart disease

Idiopathic.

CLINICAL AND LABORATORY OBSERVATIONS

As associated diseases are variable, so are the clinical manifestations. Usually the signs and symptoms of the underlying disorder overshadow those of the anemia, but on rare occasions, reduction of the hemoglobin level provides the first evidence of the existence of the primary condition (e.g. in temporal arteritis, etc).

ANEMIA: DEVELOPMENT AND SEVERITY

Typically, anemia develops during the first 1–2 months of illness and thereafter does not progress. The hematocrit is usually maintained between 25 and 40, but significantly lower values may be found in 20–30% of cases. The hemoglobin concentration and PCV generally provide an accurate reflection of the extent to which the circulating red cell mass is reduced, although in certain cases expansion of the total blood volume would mean that the reduction of red cell mass is less than the hemoglobin or PCV indicated. This is particularly likely in syndromes associated with increased levels of interleukin-6 (IL-6). IL-6 produces a dilutional anemia: expansion of the plasma volume results in a reduced hematocrit or hemoglobin concentration without changes in the circulating red cell mass.

Usually, a general correlation exists between the degree of anemia and the severity of the underlying disease. For instance, infections accompanied by pronounced fever, chills, and suppuration are associated with more severe anemia than those with fewer systemic manifestations. In infected wounds, the degree of anemia is proportional to the number of organisms present. Correlation has also been observed between the severity of the anemia and the activity of rheumatoid arthritis, judged by fever, severity of joint swelling and inflammation, and the ESR. In malignancies, anemia is more severe in patients with metastases as compared to these with localized malignancies; however, the development of anemia does not require neoplastic invasion of the bone marrow. Typically, the reticulocytes are normal or reduced in number although, rarely, they may be mildly increased.

Morphologic Features

The RBCs are usually normocytic and normochromic; however, rarely microcytosis with hypochromia may be observed (in 20–40% of cases). Hypochromia is more common than microcytosis. Hypochromia may be observed inspite of PCV remaining normal. Microcytosis when seen in these conditions is not as pronounced as seen in iron deficiency cases. Mild poilkilocytosis too may also be observed. Routine examination of the blood smear rarely reveals specific morphologic abnormalities.

Iron Metabolism

Characteristically, serum iron concentration, total iron binding capacity and transferrin saturation—all are reduced. In patients with infection, hypoferremia develops early in the course of the illness, often within 24 hours, and is observed even in acute, self-limited febrile diseases (e.g. viral fevers). When the infection is of short duration, the serum iron returns to normal and anemia does not develop in prolonged illness, the serum iron level remains low as long as the disease is active. When the disorder subsides, anemia often

is relieved before the serum iron level returns to normal. The degree of hypoferremia is related to the severity of underlying disease.

In bone marrow aspirates stained for iron; the number of sideroblasts is reduced to 5–20% of the total quantity of normoblasts (normal being 30–50%). In contrast, the amount of hemosiderin within macrophages usually is increased; exceptions probably represent cases complicated by iron deficieny.

Serum ferritin levels are useful indicators of iron status in patients without underlying chronic disorder. In patients with the anemia of chronic disease, however, the serum ferritin level indicative of adequate reticuloendothelial iron stores requires upward adjustment. Serum ferritin values usually increase in patients with inflammatory diseases and extreme elevations of serum ferritin may be nonspecific indicator of significant underlying disease. When iron deficieny coexists, the serum ferritin level falls, but may not reach values as low as those found in uncomplicated iron deficiency. A patient with chronic inflammatory disease and a serum ferritin less than 30 μg/L is certainly iron deficient, and a patient with a serum ferritin greater than 200 μg/L is certainly not iron deficient; in other circumstances. Certainty can be provided by examination of marrow by prussian blue reaction.

Other Biochemial Findings

The concentration of free erythrocytic protoporphyrin (FEP) tends to be elevated in patietns with anemia of chronic disorder (but is not as much as seen in iron deficiency). The concentrations of certain plasma proteins, such as fibrinogen, ceruloplasmin, haptoglobin, CRP, orosomucoid, C3, and amyloid A protein increase, whereas the concentrations of albumin and transferrin characteristically decrease. The increase in ceruloplasmin accounts for the increase in serum copper levels often noted in association with chronic disorders. An elevated fibrinogen level is probably the most important factor in the increased ESR. Patients with chronic illness develop accelerated protein catabolism and negative nitrogen balance associated with muscle proteolysis. Over time, this phenomenon can result in muscle wasting, increased urea excretion, weight loss, and growth impairment in children. However, protein catabolism generates amino acids that can be used by the patient as alternative energy sources or to supply substrates for biosynthetic processes related to host response. In this context, it is perhaps relevant that elevated serum levels of tumor necrosis factor (TNF) a cytokine implicated in the pathogenesis of the anemia of chronic disorders, are noted in patients with significant malnutrition.

Kinetic Characteristics

Erythrocyte survival is modestly but significantly reduced in patients with the anemia of chronic disorder. There is little evidence of a compensatory erythropoietic response to this reduction in red cell survival. The reticulocyte count is usually normal or reduced and little or no erythroid hyperplasia of the marrow is observed. Briefly said, the anemia develops because the bone marrow fails to increase red cell production sufficiently to compensate for a mild decrease in red cell life span.

Pathogenesis

The pathogenesis of anemia here is based upon (a) shortened erythrocyte survival, (b) impaired marrow response, and (c) disturbance in iron metabolism. The modest shortening of the erythrocyte survival creates an increased demand for red cell production on the marrow. Normally, the marrow could easily accommodate this demand, but in the

setting of anemia of chronic disorder, the marrow is unable to respond fully because of a combination of a blunted erythropoietin response, an inadequate progenitor response to erythropoietin, and limited iron availability.

Cytokines

In chronic disorders there is activation of macrophages and the elaboration of macrophage cytokines. These cytokines, in turn, act on other cells, such as lymphocytes, endothelial cells, and fibroblasts, to produce mediators of erythropoietic suppression, or act directly on affector cells. The central role of these cytokines suggests that the anemia of chronic disorders may be best understood as a cytokine-mediated process. The cytokines most often implicated in the pathogenesis of the anemia of chronic disorders are TNF, IL-1, and interferons. Concentrations of these cytokines have been reported to be increased in the serum or plasma of patients with disorders associated with anemia of chronic disorders, and therapeutic administration of TNF and interferon may induce anemia. IL-6, another cytokine implicated in the inflammatory and immune response, is a special case. Although increased serum IL-6 is observed in anemic patients with rheumatoid arthritis, IL-6 does not suppress erythopoiesis but rather is associated with increased plasma volume; the anemia is therefore dilutional. The association of IL-6 with hepatocyte activation may therefore explain the dilutional component of the anemia observed in liver disease.

Shortened Red Cell Survival

The rate of survival of cells from patients with arthritis, when transfused into the normal subjects, is normal, and the survival of red cell from normal individuals in the circulation of patients with arthritis is less than the normal rate. Therefore, shortened RBC survival in patients with chronic inflammatory disorders is attributed to an extracorpuscular mechanism. IL-1 and TNF are the probable causes for this. The precise mechanism by which this shortened RBC survival is produced remains unclear. Activated macrophages clear minimally damaged erythrocytes from the circulation more rapidly; this includes RBCs damaged by fever. Certain tumours may produce hemolysins, or vascular injury may permit leakage of blood into damaged tissues, leading to localized erythrocyte destruction.

Impaired Marrow Response

The possible defects in erythropoiesis fall into three categories:

1. Inappropirately low erythropoietin secretion,
2. Diminshed marrow response, and
3. Iron-limited erythropoiesis.

The erythropoietin concentration in chronic disorders is reduced when compared with proportionate anemia caused by other reasons, i.e. erythropoietin (EPO) response to anemia is blunted. It appears that this impaired EPO response is cytokine-mediated IL-1, TNFα, and transforming growth factor-β may inhibit EPO production. Another explanation is that the anemia is adaptive; that is, it reflects or reduced rate of oxygen use by tissues so that normal oxygenation is maintained despite reduced hemoglobin levels. A fancyful description is that "the hematologic factory operates at lesser capacity" possibly in order to allow diversion of substrates normally used in erythropoiesis to more immediately critical activities. A fact that supports this contention comes from the observation that certain chronic disorders are associated with decreased conversion of thyroxine (T_4) to

triiodothyronine (T_3) in peripheral tissues. As a result, serum T_3 levels are reduced despite minimal evidence of clinical hypothyroidism (the sick euthyroid syndrome). These findings provide a basis for reduced oxygen consumption at the tissue level. However, changes that ordinarily signify erythrocyte adaptation to tissue hypoxia, such as increased erythrocyte 2,3-DPG levels and decreased hemoglobin oxygen affinity, are observed in the anemia of chronic disorders.

Although the EPO levels of patients with anemia of chronic disorders are lower than those observed in equally iron-deficient individuals, they are still higher than are observed in normal individuals who are not anemic. This implies that inhibition of EPO production cannot entirely account for the impaired erythropoiesis associated with anemia of chronic disorders, and that the erythroid progenitors themselves exhibit an abnormal response to erythropoietin. TNF, IL-1 and interferon have all been reported to inhibit erythropoiesis *in vivo* and *in vitro*. Treatment with recombinant human erythropoietin can correct the anemia of chronic disorders in many cases.

Abnormal Iron Metabolism

It has been proposed that lack of iron for erythropoiesis contributes to the inadequate marrow response in the anemia of chronic disorders. Evidence of a functional iron deficieny in this syndrome includes erythrocyte microcytosis, increased FEP, reduced transferrin saturation, and decreased marrow sideroblasts.

The major contributor to hypoferremia in patients with the anemia of chronic disorders is probably a shift of iron from a transferrin-bound, available state to a ferritin-bound storage state. Iron absorption appears to be normal but iron tends to remain in the mucosal cells and in hepatocytes. Macrophages, the major site from which iron is obtained for erythropoiesis, also exhibit increased iron storage. Macrophage iron becomes available for erythropoiesis through two mobilization pathways: a rapid pathway associated with almost immediate return of the iron retrieved from senescent red cells, and a slower pathway, consisting of iron mobilized from storage. In the anemia of chronic disorders, the slower pathway predominates and iron tends to accumulate.

Apoferritin is normally synthesized in response to increased intracellular iron concentration. One possibility is that, like acute phase proteins, excess apoferritin is made in inflammatory and malignant conditions, and the surplus binds a larger than usual amount of iron entering the cells. In effect, such a mechanism would divert iron from the rapid to the slow pathway of iron release. Studies of cytokine effects on iron mobilization support this hypothesis.

The lactoferrin hypothesis is another approach to explaining iron abnormalities in the anemia of chronic disorders. Lactoferrin is a transferrin like protein found in neutrophil specific granules. It is released from the neutrophil during phagocytosis or stimulation by IL-1. At low pH, lactoferrin binds iron more avidly than does transferrin. Lactoferrin bound iron is not immediately available for erythropoiesis, but rather binds to specific receptors on macrophages (particularly in the liver and spleen) and is endocytosed and subsequently incorporated into ferritin. Thus lactoferrin transfers iron from its transferrin bound, circulating state to a storage state, from which it can be rapidly mobilized.

In addition to decreased availability of iron, erythroid progenitors may also be unable to fully use the iron available to them. Erythroblasts from anemic patients with rheumatoid arthritis; express fewer surface transferrin receptors (TfRs) than do erythroblasts from normal individuals. These TfRs also exhibit lower binding affinity for transferrin.

Furthermore, acute phase reactants, such as α-1-antitrypsin, impair transferrin binding to erythroblasts and also inhibit transferrin internalization.

Anemia in Patients with Cancer

Besides factors of anemia of chronic disorders, other factors may be operative too. Erythroid precursors may be displaced from marrow by metastatic tumor, tumor-induced fibrosis, or tumor-associated marrow necrosis. The treatment of cancer can also produce or exacerbate anemia by a variety of mechanism, including impaired erythropoietin production and cytotoxic effects on erythroid precursors.

Diagnosis

It has been noted that diagnosis of anemia of moderate degree, as is commonly observed in the anemia of chronic disorders is often missed. The anemia of chronic disorders should be considered in anemic patients with associated inflammatory, infections, or neoplastic states. The diagnosis is confirmed by demonstrating hypoferremia with adequate reticuloendothelial iron stores in a patient with an appropriate clinical syndrome classically, the serum transferrin is either low or low normal. The major differential diagnosis is iron deficiency anemia. This is important. The diagnosis of iron deficiency anemia mandate identification of a source of blood loss. Mislabeling the diagnosis can cost the patient heavily.

Treatment

Most importantly, one has to treat the under lying disorder. The anemia itself is rarely an important clinical problem. Thus, direct approaches to correction of the anemia are rarely necessary. Where necessary, recombinant human erythropoietin is effective and safe, but expensive. Because most patients are not symptomatic from anemia, symptomatic benefit is rarely observed. Its use should probably be reserved for patients who actually require transfusion, or for those who wish to donate blood for autologous transfusion at a later date.

Despite reports that iron given parenterally may bring about some improvement, such therapy is not recommended on account of attendent risks when comapred with low likelyhood of benefits. Oral iron replacement is likely to be useful only in patients who have concurrent iron deficiency, and then only for the component of anemia caused by iron deficiency. Iron does not correct the anemia of chronic disorders *per se*.

NORMOCYTIC AND NORMOCHROMIC ANEMIAS (FLOWCHART 7.1)

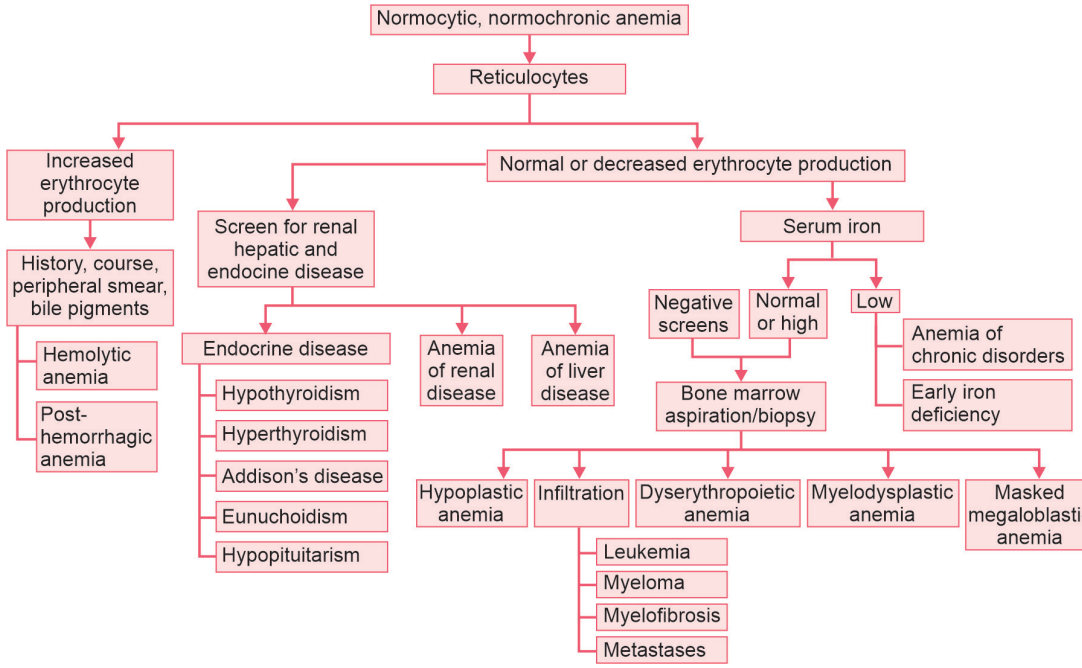
A normocytic anemia is defined as an anemia in which the MCV is within normal range, i.e. 76–96 fL. Most normocytic anemias are normochromic. However, in some cases there is a mild-to-moderate degree of hypochromia.

ETIOLOGICAL CLASSIFICATION OF NORMOCYTIC ANEMIA

I. Blood loss
   - Acute blood loss
   - Chronic blood loss (prior to development of iron deficiency)

II. Disorders causing depression of bone marrow functions
   - Infection
   - Renal failure
   - Disseminated malignancy

Flowchart 7.1: Algorithm for investigation of a patient with normocytic, normochromic anemia



- Liver disease
- Collagen diseases
- Bone marrow infiltration—leukemia, malignant lymphoma, multiple myeloma, myelosclerosis, metastatic bone carcinoma
- Aplastic anemia
- Endocrine disorder—myxoedema, hypopituitarism.
- Addison's disease
- Protein malnutrition
- Scurvy

III. The hemolytic anemias

IV. The physiological anemia of pregnancy.

SUMMARY OF THE INVESTIGATIONS OF A PATIENT WITH NORMOCYTIC ANEMIA

HISTORY

Usual, plus with special reference to:

- Rate of onset
- Blood loss
- Alimentary symptoms
- Bleeding tendency
- Nocturia
- Bone pain
- Symptoms suggestive of myxoedema or hypopituitarism
- Alcoholism.

EXAMINATION

Usual, plus with special reference to:

- Skin petechiae or ecchymoses
- Conjunctivae: Icterus, hemorrhage
- Abdomen: Hepatomegaly, splenomegaly, tenderness, mass, ascites.
- Signs associated with renal insufficiency: Hypertension, retinitis, proteinuria
- Signs of localized infection
- Pyrexia
- Tourniquet test.

BLOOD EXAMINATION

Take special notice of:

- Aniocytosis and poikilocytosis
- TLC and DLC
- Reticulocyte count
- Immature white and red cells
- ESR.

SPECIAL INVESTIGATIONS

- Fecal occult blood (GI bleeding)
- Barium meal or enema (GI bleeding)
- Microscopic examination of urine (chronic renal infection, hematuria)
- Blood urea (chronic renal insufficiency)
- Skeletal X-ray (secondary carcinoma of bone, multiple myeloma, malignant lymphoma, myelosclerosis)
- Liver function tests (liver disease)
- Bone marrow aspiration (aplastic anemia, subleukemic leukemia, multiple myeloma, secondary carcinoma of bone)
- Bone marrow trephine biopsy (aplastic anemia, metastatic carcinoma, lymphoma, myelosclerosis)
- Blood culture (bacterial endocarditis)
- PBI and other thyroid function tests
- LE cell test (SLE), ANA, anti ds DNA
- Special investigations for hemolytic disease.

CHAPTER

8

Hemolytic Anemias

DEFINITION

A hemolytic anemia is one that ensues from an increase in the rate of red cell destruction. The normal lifespan of red cells (100–200 days) is shortened by various degrees in this group of anemias. To put it in other words—RBCs die in their childhood or adolescence or middle age without reaching the age of senescence.

In the broadest sense, such a definition includes a number of common but kinetically complex anemias, such as the anemias of chronic disorders and of renal disease, anemia associated with vitamin B_{12} and folate deficiency and even iron-deficiency anemias. The rate of red cell destruction associated with these disorders is modestly increased, but not to a degree that would by itself lead to anemia. The anemia develops because of a more fundamental pathogenetic factor namely inability of the marrow to increase red cell production sufficiently to compensate for the shortened survival of the red cells (relative marrow failure). These illnesses have a hemolytic component, but it would be misleading to include them with the hemolytic anemias. As commonly understood, the term hemolytic anemia or better still hemolytic disorders, is limited to conditions in which the rate of red cell destruction is accelerated and the ability of bone marrow to respond to the stimulus of anemia is unimpaired.

The distinction between frankly hemolytic anemias and those associated with relative marrow failure is better understood quantitatively. Under maximal stimulation, the normal marrow is capable of undergoing hyperplasia until its production rate increases about 6–8 times. With optimal marrow compensation, the survival of red cells in the circulation can theoretically decrease from the normal 120 days to as low as 15–20 days without anemia developing. Such an increase in both destruction and production of erythrocytes can result in a compensated hemolytic state without anemia being present, the so-called compensated hemolytic anemia. When red cell survival is so short that anemia develops despite a vigorous erythropoietic response, however, the term hemolytic anemia is appropriate.

The premature destruction of the red cells may result from two basic defects: (1) an intracorpuscular (intrinsic) abnormality of the red cells which renders them more susceptible to the normal mechanisms of cell destruction. The fault lies in the cells themselves. Can this be proved experimentally? Yes—Normal compatible red cells when transfused to such a patient survive for a normal time, but the patient's cells when transfused into a normal recipient die early; (2) an extracorpuscular (extrinsic) abnormality due to development of an abnormal hemolytic mechanism. The fault lies in the patient's plasma or tissues; the red cells are primarily normal. Normal compatible red cells transfused into a patient with an extracorpuscular abnormality are prematurely destroyed, but the patient's cells transfused

into a normal recipient survive for more or less a normal time. Hemolytic anemias can, therefore, be grouped under two broad headings:

1. *Hemolytic anemias due to corpuscular defect (intracorpuscular or intrinsic abnormality):* These are mainly congenital. The basic defect may be in any of the three main components of the cell, i.e. the membrane, the hemoglobin molecule and the enzymes concerned with cell metabolism.
2. *Hemolytic anemias due to an abnormal hemolytic mechanism (extracorpuscular or extrinsic abnormality):* These are acquired. The hemolysis may result from either an immune or nonimmune mechanism.

Another way of classifying these anemias is based upon the site of hemolysis that is whether the destruction is within the circulation (intravascular) or within tissue macrophags (extravascular). The manifestation such as hemoglobinuria, hemoglobinemia and hemosiderinuria; it is recognized easily, thereby limiting the diagnostic possibilities. However, most hemolytic diseases are characterized by extravascular red cell destruction, detection of the mode of hemolysis still leaves a large number of diagnostic possibilities (Table 8.1).

Hemolytic Anemias Characterized by Mainly Intravascular RBC Destruction

Paroxysmal nocturnal hemoglobinuria, disorders associated with RBC fragmentation, certain immunohemolytic anemias

- Transfusion reactions
- Paroxysmal cold hemoglobinuria
- Some cases of idiopathic autoimmune hemolytic anemia

Those associated with certain infections

- Blackwater fever in falciparum malaria
- Clostridial sepsis

Those caused by certain chemical agents

- Intravascular administration of distilled water
- Snake and spider venoms
- Arsine poisoning
- Acute drug reactions in association with G6PD deficiency.

Thermal injury

Compensated Hemolytic Anemia

This is a state where despite the hemolysis, anemia does not occur. The greatly expanded (hyperplastic) bone marrow provides an adequate supply of red cells so that anemia does not occur.

Table 8.1: Relative values for rates of hemoglobin production and destruction

	Hb. Concentration g/dL	*Total (g) Circulating Hb*	*RBC Survival days*	*Hb Produced and destroyed every (g/day)*
Normal	16.7	800	120	6.7
Relative marrow failure	10.0	480	60	8.0
Compensated hemolytic state	16.7	800	20	40.0
Hemolytic anemia	10.0	480	12	40.0

Assuming that a normal adult has about 4.8 L of circulating blood volume (splenic pool excluded).

$$\text{Hb produced and destroyed (g/day)} = \frac{\text{Total circulating Hb (g)}}{\text{RBC survival (days)}}$$

CLASSIFICATION

Classification based upon intra- and extracorpuscular defects.

INTRACORPUSCULAR

Hereditary or Congenital

Membrane Defects

- Hereditary spherocytosis
- Hereditary elliptocytosis
- Hereditary stomatocytosis
- Hereditary pyropoikilocytosis
- Hereditary xerocytosis

Hemoglobin Defects

- Hemoglobinopathies
- Sickle cell disease
- Hb CDE, etc.
- Unstable Hb disease

Thalassemia

- β-thalassemia major
- Hb H disease.

Enzyme Defects

Non-spherocytic Congenital Hemolytic Anemia

- Due to phosphokinase deficiency or other EM pathway enzyme defects.
- Due to G6PD deficiency or other pentose phosphate pathway enzyme defects.
- Drug induced hemolytic anemia
- Favism.

Acquired

- Paroxysmal nocturnal hemoglobinuria.

EXTRACORPUSCULAR

Acquired

Immune Mechanism

- Autoimmune hemolytic anemia
  - Warm antibody type
  - Cold antibody type
- Hemolytic disease of the newborn
- Incompatible blood transfusion
- Drug-induced hemolytic anemia.

Nonimmune Mechanism

- Mechanical hemolytic anemia
- Cardiac hemolytic anemia
- Microanglopathic hemolytic anemia
- March hemoglobinuria

Miscellaneous

- Hemolytic anemia due to direct action of drugs/chemicals
- Hemolytic anemia due to infection (*Clostridia welchii*)
- Hemolytic anemia due to burns
- Lead poisoning.

ETIOPATHOGENETIC CLASSIFICATION OF HEMOLYTIC DISORDERS

Inherited Hemolytic Disease

Defects in Erythrocyte Membrane

1. Hereditary spherocytosis
2. Hereditary elliptocytosis
   a. Mild (common) variant
   b. With neonatal poikilocytosis
   c. Pyropoikilocytosis
   d. Spherocytic elliptocytosis
   e. Stomatocytic (Melanesian) elliptocytosis
3. Abetalipoproteinemia (acanthocytosis)
4. Hereditary stomatocytosis
5. Lecithin-cholesterol acyltransferase (LCAT) deficiency
6. High phosphatidylcholine hemolytic anemia
7. Rh_{null} disease
8. McLeod phenotype

Deficiency of Erythrocyte Glycolytic Enzymes

1. Pyruvate kinase deficiency
2. Phosphoglucose isomerase deficiency
3. Phosphofructokinase deficiency
4. Triosephosphate isomerase deficiency
5. Hexokinase deficiency
6. Phosphoglycerate kinase deficiency
7. Aldolase deficiency
8. Diphosphoglycerate mutase deficiency

Abnormalities of Erythocyte Nucleotide Metabolism

1. Pyrimidine-5′-nucleotidase deficiency
2. Adenosine deaminase excess
3. Adenosine triphosphatase deficiency
4. Adenylate kinase deficiency

Deficiencies of Enzymes Involved in the Pentose Phosphate Pathway and in Glutathione Metabolism

1. Glucose-6-phosphate dehydrogenase (G6PD)
2. Glutamyl-cysteine synthetase
3. Glutathione synthetase
4. Glutathione reductase

Defects in Globin Structure and Synthesis

1. Unstable hemoglobin disease
2. Sickle cell anemia

3. Other homozygous hemoglobinopathies (CC,DD,EE)
4. Thalassemia major
5. Hemoglobin H disease
6. Doubly heterozygous disorders (such as hemoglobin SC disease and sickle thalassemia).

Acquired Hemolytic Anemias

Immunohemolytic Anemia

1. Transfusion of incompatible bood
2. Hemolytic disease of the newborn
3. Autoimmune hemolytic anemia caused by warm-reactive antibodies
   a. Idiopathic
   b. Secondary or symptomatic
      i. Virus and *Mycoplasma* infections
      ii. Lymphosarcoma CLL
      iii. Other malignant disease
      iv. Immune deficiency states
      v. SLE and other autoimmune disorders.
   c. Drug induced
4. Autoimmune hemolytic anemia caused by cold-reactive antibodies
   a. Cold hemagglutinin disease
      i. Idiopathic
      ii. Secondary
   b. Paroxysmal cold hemoglobinuria.

Traumatic and Microangiopathic Hemolytic Anemias

1. Prosthetic valves and other cardiac abnormalities
2. Hemolytic–uremic syndrome
3. Thrombotic thrombocytopenic purpura
4. Disseminated intravascular coagulation
5. Associated with immunologic phenomena (such as graft rejection and immune complexes)
6. Cancer
7. Naked stint syndrome.

Infectious Agents

1. **Protozoa:** Malaria, toxoplasmosis, leishmaniasis, trypanosomiasis, babesiosis
2. **Bacteria:** Bartonellosis, clostridial infection, cholera, typhoid fever, and others.

Chemicals, Drugs and Venoms

1. Oxidant drugs and venoms
2. Nonoxidant drugs and chemicals
3. Associated with hemodialysis and uremia
4. Venoms.

Physical Agents

1. Thermal injury.

F. Hypophosphatemia

G. Paroxysmal nocturnal hemoglobinuria

H. Spur cell anemia in liver disease

I. Vitamin E deficiency in newborns.

CLINICAL MANIFESTATIONS

The clinical manifestations of hemolytic disorders depend upon their duration and severity. Thus, chronic congenital hemolytic anemia can usually be distinguished from acute acquired hemolytic anemia on clinical grounds. When the onset of acquired hemolytic anemia is insiduous, however, the distinction may be more difficult.

CHRONIC CONGENITAL HEMOLYTIC ANEMIA

The significant clinical features of congenital hemolytic anemia are related to anemia, jaundice, the occurrence of crises, splenomegaly, and the development of gallstones. Less common features include chronic leg ulcers and bony abnormalities.

Degree of Anemia

Severity of disease varies from one patient to another and even among patients with the same illness. Severe disease is ordinarily detected shortly after birth or within the first year of life. Severe pallor and the cardiovascular manifestations of anemia are the usual findings; the latter may be serious enough to require blood replenishment by way of blood transfusions.

Quite often bone marrow compensates to a greater or lesser extent the loss of red cells. Often the patients accommodate remarkably well to the anemia, and may exhibit few symptoms. A moderately pallid/sallow complexion may be the only feature under such circumstances, detection often is delayed until later in childhood.

Lastly, some patients may have no anemia at all. The disease may then remain undiagnosed until later in adult life unless jaundice, a crises, or complications of cholelithiasis draw attention to the condition. Often, such cases are discovered only in the course of a family study.

Jaundice

Sometimes intense jaundice with impending kernicterus may be observed in neonatal period, it may simulate immunohemolytic anemia necessitating exchange transfusion. In older children or adults with congenital hemolytic anemia, icterus is absent or mild enough to pass unnoticed. History, if taken carefully, may elicit episodes of jaundice associated with trivial infections or unusual exertion. Often slight scleral icterus is the only sign of hemolytic disease and the description is more yellow than sick has been appropriately applied to such patients. The jaundice of hemolytic disease is acholuric; the bilirubin, being unconjugated is not excreted in the urine. Also pruritus is absent. These features help in differentiating it from hepatobiliary system disease.

Crises

In chronic congenital hemolytic anemia, there is a delicate balance between the rates of destruction and production. Disruption of this balance can lead to rapid and dramatic falls

in blood hemoglobin level, producing a crises. The most common type of crises results from transient failure of red cell production, and is called an aplastic crises. Most of these (if not all) are caused by infection with type B_{19} human parvovirus (HPV). The same virus also causes erythema infectiosum, a common childhood exanthem, and may cause an acute polyarthropathy syndrome in adults as well as aseptic meningitis and fetal death with hydrops fetalis. HPV infection may occur sporadically or in an epidemic form. Modes of infection are thought to be fecooral, oral–oral and respiratory, with an incubation period lasting 6–12 days. The most susceptible age group is 5–10 years old school-going children. Most reports of aplastic crises involve patients with sickle cell anemia, but they also occur in patients with β-thalassemia, pyruvate kinase deficiency, and hereditary deficiency.

Aplastic crises resulting from HPV infection begins with nonspecific manifestations of infectious disease such as fever, chills, lethargy and malaise. Sore throat, cough, abdominal pain, diarrhea and vomiting are common. About 25% of these develop a faint, maculopapular rash. Hematologic abnormalities follow about 4 days later (range being 2–17 days). Blood hemoglobin concentration falls to about 2–6 g/dL sometimes to life-threatening levels and the reticulocyte count falls abruptly, usually to less than 1%. The severity of crises depends upon severity of hemolytic process.

In most cases, leucocytes and platelets are unaffected, but neutropenia and thrombocytopenia can occur and, rarely, may be severe. The bone marrow is cellular but erythroid hypoplasia is the chief finding, the more mature erythrocyte precursors tend to disappear, giant pronormoblasts may be observed. The crises lasts about 6–8 days. Recovery is heralded by reticulocytosis, the disappearance of fever and, sometimes by leukocytosis and thrombocytosis. This entire episode from onset of symptoms to reappearance of reticulocytes, lasts about 10–12 days in subjects with normal immunologic responses, but may persist much longer in patients with defective or suppressed immune systems. HPV infection also induces erythroid aplasia in normal subject but the hemoglobin concentration falls only 1–2 g/dL, when erythrocyte survival is normal, such changes go unnoticed.

HPV infection can cause anemia in conjunction with other nonhemolytic disorders, such as AIDS, iron deficiency and often bone marrow or renal transplantation. It may also cause a congenital anemia often with transplacental passage. A single infection with HPV confers life-long immunity. The virus probably Acts by infecting replicating cells in erythroid line. It inhibits growth of CFU-Es (colony-forming units-erythroid) and to a lesser extent, BFU-Es (burst-forming units-erythroid), but apparently does not attack other hematologic stem cells. In aplastic crisis blood transfusions are often required.

In some cases, an abrupt increase in the rate of red cell destruction may occur, possibly because of increased splenic activity (hemolytic crises). In such episodes, the degree of jaundice and the reticulocyte count increase and spleen may enlarge. A third type of crises comes about because of complicating folate deficiency (megaloblastic crises) to which patients with chronic hemolysis appear to be particularly prone. The onset of megaloblastic crises tends to be more gradual than that of aplastic or hemolytic crises and is unrelated to complicated infections.

Splenomegaly

The spleen typically is enlarged (usually mild to moderate) with congenital hemolytic anemia. At times splenomegaly may be the first finding on a routine physical examination. Spleen may also attract attention because of a sense of heaviness in the region or because of pain.

Cholelithiasis

Sometimes symptoms of gallbladder disease may be the first manifestations of hemolytic process. Gallstones typical of a hemolytic anemia are so-called black pigment stones. They differ from brown pigment stones and cholesterol stones, neither of which is associated with hemolysis. The color of the stone is explained by its content of an insoluble black pigment that probably is a cross-linked network polymer of bilirubinate. It constitutes 40% of the stone by weight. Variable amount of other calcium salts are also present black pigment stones usually are amorphous, brittle, and less than 10 mm in diameter. About 40–80% of them are radiopaque. Oral cholecystography demonstrates their features; multiple, nonfloating, irregularly-shaped, and often radiopaque. The prevalence of cholelithiasis in patients with hemolytic anemia increases with age and with the intensity of hemolytic process.

Cholelithiasis is slightly more common in women with hemolytic disease than in men. The increase in bilirubin and calcium in bile of these patients favor supersaturation of the bile with calcium bilirubinate. Stone formation may begin in the glandular crypts of the gallbladder mucosa; where microscopic precipitates of calcium bilirubinate are deposited. The mucin glycoprotein at these sites provides a framework on which the stone can grow. Polymerization occurs later in the solid state.

Leg Ulcers

Chronic leg ulcers are particularly characteristic of hereditary spherocytosis and sickle cell anemia. They are also seen in other chronic hemolytic anemias. About 5–10% of sickle cell anemia cases suffer from leg ulcers. Lower rates are observed with sickle cell-thalassemia combinations and in sickle-hemoglobin-C disease. Ulcers are observed most often in males over the age of 20 years. The ulcers are often bilateral and tend to involve the areas overlying or proximal to the medial or lateral malleoli. When severe, they may extend a considerable distance up the leg and may completely surround it. They tend to be chronic or recurrent and, upon healing, leave the skin indurated and pigmented. Interruption of the hemolytic process, as by splenectomy in hereditary spherocytosis, induces healing of ulcers.

Skeletal Abnormalities

In severe hemolytic anemia during active phases of growth and development, the expansion of the erythroid bone marrow may lead to a tower-shaped skull, thickening and striation of frontal and parietal bones, maxillary and dental abnormalities, and other distortions of bony structures. Such abnormalities are particularly characteristic of severe thalassemia major and may also be seen in patients with sickle cell anemia and in exceptional patients with other forms of congenital hemolytic anemia.

ACQUIRED HEMOLYTIC ANEMIA

In case hemolytic anemia develops acutely, as often the transfusion incompatibility reaction, ingestion of an oxidant drug by patients with G6PD deficiency, the symptoms may suggest an acute febrile illness. Some cases of autoimmune hemolytic anemia, thrombotic thrombocytopenic purpura, and other hemolytic disorders may also begin abruptly. Aching pains in the back, abdomen or limbs are common, as are headaches malaise, vomiting, shaking chills and fever. Abdominal pain and accompanying muscular spasm and rigidity may simulate the signs of an acute abdomen requiring surgical intervention. Profound prostration and shock may develop, followed by oliguria and anuria. Pallor, jaundice, tachycardia and other symptoms of severe anemia may be prominent.

More often, acquired hemolytic anemia begins insiduously, developing gradually over a period of weeks or months. Cardiovascular adjustments to the anemia may be enough and patients may have few symptoms. The clinical picture may then be similar to that described for congenital hemolytic disease. Pallor, scleral icterus, or a jaundiced complexion may be the first evidence of illness, and often first noticed by friends or relatives when anemia is more severe, the patient typically complains of weakness, fatigability, dyspnea, or other cardiovascular symptoms. As in congenital hemolytic anemia, the course may be interrupted by aplastic crises.

In other cases, the clinical features may be dominated by the manifestations of an underlying disease of which the hemolytic anemia is one manifestation. For instance, signs and symptoms of lymphoma, lupus erythematosus, or mycoplasma pneumonia may overshadow those of the associated hemolytic process.

LABORATORY MANIFESTATIONS

These findings can be divided into three groups:

a. Those related to the increase in erythrocyte destruction
b. Those related to the compensatory increase in the rate of erythropoiesis,
c. Those found only in particular varieties of hemolytic anemia, which therefore are useful in differential diagnosis.

Laboratory Signs of Accelerated Red Cell Destruction

- Decreased erythrocyte lifespan
- Increased catabolism of hem
  - Increased serum unconjugated bilirubin level
  - Increased endogenous carbon monoxide production
  - Increased rate of bilirubin production
  - Increased rate of urobilinogen excretion
- Increased serum lactate dehydrogenase (LDH) activity
- Absence of serum haptoglobin
- Reduced glycosylated hemoglobin
- Sign of intravascular hemolysis
  - Hemoglobinemia
  - Hemoglobinuria
  - Hemosiderinuria
  - Methemalbuminemia
  - Reduced serum hemopexin level
- Fall in blood hemoglobin level at a rate greater than 1.0 g/dL/week.

Signs of Excessive Red Cell Destruction

Erythrocyte Survival

Ideal method for testing this is based on random labelling with ^{51}Chromium. As measured with this labelling technique, the half disappearance time (t ½ Cr) is usually reduced in hemolytic disease, but the degree of reduction varies from one patient to another, even among those with the same disease. On the average, t ½ Cr is reduced to about one-half the normal value; however, because of the nonlinear relationship between t ½ Cr and erythrocyte life span, the reduction in the latter is greater than might be supposed. Thus, for example, a 50% reduction in t ½ Cr corresponds to an erythrocyte life span of about 30 days, or 25% of the normal. The t ½ Cr tends to be lowest in patients with the greatest

degree of anemia, but the correlation is not close. This investigation is expensive and time-consuming and therefore should be used in evaluating patients with especially difficult diagnostic problems.

Catabolism of Heme

In hemolytic disease, the heme moiety of hemoglobin is catabolized at a much accelerated rate, and excretion of the main heme catabolites— bile pigments and carbon monoxide—is proportionately increased. These changes provide clinically useful signs of hemolysis.

Serum Bilirubin

The amount of circulating bilirubin depends upon (a) the rate of its production, and (b) the rate of its excretion by the liver. So, it is not a reliable index of the rate of red cell destruction. Occasionally, it falls within normal range despite brisk hemolysis.

The increased serum bilirubin level in hemolysis almost always consists of unconjugated pigment. The conjugated fraction remains within normal limits and no bilirubin is evident in the urine.

Conditions Associated with Unconjugated Hyperbilirubinemia

- Excessive heme catabolism
  - Hemolytic anemia
  - Ineffective erythopoiesis
  - Extravasation of blood into tissues or body cavities
- Defective bilirubin conjugation
  - Complete deficiency of glucoronyl transferase (Crigler–Najjar syndrome)
  - Partial deficiency of glucuronyl transferase
  - Inhibition by certain steroids (e.g. pregnanediol)
- Lucey–Driscoll syndrome (transient familial hyperbilirubinemia)
- Breast–milk jaundice
- Pregnancy
- Mixed pathogenesis (decreased uptake and defective conjugation)
  - Neonatal hyperbilirubinemia
  - Drug-induced hyperbilirubinemia
- Flavaspidic acid
- Novobiocin
- Synkavite (menadiol sodium diphosphate)
- Estrogens
- Of uncertain pathogenesis
  - Gibert's syndrome
  - Associated with some cases of hepatitis
  - High altitude exposure
  - Fasting
  - Excercise

Rate of Heme Catabolism

Determinations of the rate of endogenous carbon monoxide production or bilirubin turnover provide accurate assessments of the rate of heme catabolism. As on date, however, these methods are far too complex for use by routine clinical laboratory. Quantitation of fecal urobilinogen excretion provides a more sensitive index of hemolysis than does the

serum bilirubin level; however, they necessitate the accurate collection and processing of timed fecal specimens. The hemolytic index is a ratio of urobilinogen excretion to the estimated circulating hemoglobin (Hb) mass. The normal value being 11–21 mg/day/100 g Hb. In hemolytic anemia average value being 255 mg/day/100 g Hb. Because the formation of urobilinogen depends on the presence of intestinal bacteria, falsely low values are to be expected in patients receiving broad spectrum antibiotics.

Lactate Dehydrogenase (LDH)

Serum LDH activity markedly increases in hemolytic anemia. In hemolytic anemia the isoenzyme LDH-2 predominates, whereas in megaloblastic anemia (LDH activity is higher than hemolytic anemia) LDH 1 predominates. The increase in LDH probably results from liberation of the erythrocyte enzyme into plasma during hemolysis.

Other causes of raised LDH activity are: (1) myocardial infarction, (2) pulmonary infarcion, (3) shock, (4) chest-wall trauma, (5) acute pancreatitis, 6) visceral infarction, (7) carcinomatosis, (8) acute and chronic renal disease, (9) muscle disorders, (10) lymphomas, and (11) acute leukemia. The utility of LDH evaluation is limited by this lack of specificity.

Disappearance of Haptoglobin

Hemoglobin, on entering the plasma, binds to haptoglobin and hepatocyte removes the complex. Consequently haptoglobin values fall to low levels in individuals with hemolytic disease. However, utility of haptoglobin estimation is limited by the fact that haptoglobin is an acute phase reactant, the synthesis of which increases in response to inflammatory, infections, or malignant disease.

The quantity of plasma haptoglobin is measured by using methods based on the peroxidase activity of hemoglobin-haptoglobin complex, by electrophoresis, chromatography or by immunonephalometry, which is the most sensitive and least arduous of the methods.

Despite the intravascular site of haptoglobin function, this protein becomes depleted not only in association with intravascular hemolytic anemias, but also in association with conditions characterized by predominantly extravascular hemolysis, such as sickle cell anemia, hereditary spherocytosis, hereditary elliptocytosis, and pyruvate kinase deficiency. In hereditary spherocytosis, the characteristically reduced haptoglobin levels are restored after splenectomy. Haptoglobin also disappears in megaloblastic anemia and other conditions in which the hemolysis is intramedullary (ineffective erythropoiesis). The sensitivity of low haptoglobin (in relation to hemolysis) is 83% and specificity is 96%. A low haptoglobin level indicates an 87% probablity of hemolytic disease. Despite these facts, the utility of haptoglobin measurement for the purpose of detecting hemolysis is not widely accepted. Values also may fall in association with liver disease because of impaired synthesis, and in hereditary deficiency of the protein. Furthermore, complicating infections as well as inflammatory or malignant conditions can normalize the haptoglobin value in patients with hemolytic disease.

Glycosylated Hemoglobin (Hb A1)

Hb A1 values are regularly reduced in people with hemolytic disease. The glycosylated hemoglobin level perhaps is related to the magnitude of the hemolytic process over the previous 4–8 weeks. The evaluation of Hb A1 is a useful measure of hemolysis, provided that diabetes mellitus and posthemorrhagic anemia can be excluded.

Signs of Intravascular Hemolysis

When RBCs are destroyed within the circulation, and also when extravascular destruction is so rapid that it exceeds the capacity of the macrophage system, hemoglobin is released into the plasma. The disposal of hemoglobin and its heme group occurs by several mechanisms, and characteristic laboratory abnormalities are observed.

Hemoglobinemia

Normal value of plasma hemoglobin is less than 1 mg/dL. Plasma usually appears visibly red when hemoglobin exceeds 50 mg/dL. For lower plasma hemoglobin levels detection—the benzedine reaction is useful, however, for values above 100 mg/dL the hemoglobin can be directly measured by the cyanmethemoglobin method. In intravascular hemolysis values of 1000 mg/dL or more are common.

Hemoglobinuria

When plasma hemoglobin exceeds the haptoglobin binding capacity, hemoglobin dimers are excreted in the urine, resulting in hemoglobinuria. Urine that contains hemoglobin ranges from faint pink to deeper red, or even to almost black, similar to a cola beverage. The colour depends upon the concentration of hemoglobin as well as on the oxidation state and degree of dissociation of the heme group.

Hemoglobinuria can be differentiated from hematuria (whole RBCs in urine) by microscopic examination of a freshly voided urine specimen.

Other causes of red colored urine are ingestion of certain drugs (pyridium, antipyrine) or food (beets), or because of porphyrinuria or myoglobinuria. Of these various red urinary pigments, only hemoglobin and myoglobin produce a positive reaction in the commonly available tests for occult blood, which are based on benzidine or orthotolidine reactions.

Hemoglobinuria must be differentiated from myoglobinuria which occurs as a result of massive muscle injury by trauma, electric shock, arterial thrombosis, certain toxins ("haff" disease), Malayan sea-snake bite, idiopathic myoglobinuria, myophosphorylase deficiency (McArdle disease), drug induced—statins used for lowering cholesterol, and other causes. Myoglobin is a heme pigment of low molecular weight (17,000 Da); it is not bound by haptoglobin and therefore does not accumulate to an appreciable extent in the plasma. Thus inspection of plasma can help to distinguish myoglobinuria from hemoglobinuria, the presence of red color favoring the latter. More precise identification is obtained by electrophoresis on paper, starch, or acrylamide gels or by spectrophotometry. Spectrophotometry is facilitated by treatment with carbon monoxide because a greater divergence exists between the bands of caraboxyhemoglobin and carboxymyoglobin than between oxygenated forms. Unlike hemoglobin myoglobin remains in solution after 80% saturation of the urine with ammonium sulphate.

Urine Iron Excretion

Hemoglobin in the glomerular filtrate is partially reabsorbed by the proximal tubular cells and the hemoglobin iron is incorporated into ferritin and hemosiderin. Subsequently, the iron containing tubular cells are sloughed into the urine. Hemosiderinuria and increased urinary iron excretion, therfore, constitute reliable evidence that hemoglobinemia has occurred in recent past. After an acute episode several days may go before increased iron excretion can be detected. Moreover, the abnormality may persist much longer, after the acute episode has terminated. In most chronic intravascular hemolysis conditions, increased iron excretion is a constant finding, whereas hemoglobinuria occurs only

intermittently. The only nonhemolytic causes in which increased iron excretion is found are hemochromatosis and the nephrotic syndrome.

Hemosiderinuria may be detected by means of a qualitative test based on the Prussian blue reaction. Alternatively, urinary iron content may be determined spectrophotometrically after wet digestion of a measured urine specimen: the normal value for urinary iron excretion is less than 0.1 mg/day. In a variety of disorders associated with intravascular hemolysis, urinary iron values increase to between 3 and 11 mg/day. Normal or nearly normal values are found in patients with pernicious anemia and in hereditary spherocytosis. Thus, urinary iron determination is a useful and consistent sign of intravascular hemolysis. It may not reflect the current ongoing clinical situation, however, because of its persistence after termination of the process.

Methemalbumin and Hemopexin

Hemoglobin in plasma is readily oxidized to methemoglobin, from which the heme group easily detaches. The liberated heme binds to hemopexin and also to albumin, forming methemalbumin. Hemopexinheme and methemalbumin impart a coffee-brown color to plasma. With either combination, spectral absorption band is observed at 620–630 nm, which, unlike a similar band in methemalbumin, does not disappear if hydrogen peroxide is added. With the addition of ammonium sulfide, at 620–630 nm, band disappears and a band at 558 nm forms (Schumm test).

The presence of these pigments strongly suggests intravascular hemolysis, but they have also been observed in association with hemorrhagic pancreatitis. Although hemopexin can be depleted in the course of serving its function, depletion occurs less regularly than as occurs with haptoglobin. Low hemopexin values usually indicate that the hemolytic anemia is severe; values are often near normal if illness is mild. Hemopexin tends to be absent or scant in mildly affected patients with unstable hemoglobins that lose heme as they denature intracellularly. Low hempexin values are also characteristic of thalassemia major, sickle cell anemia, and the hemolytic anemia that follows cardiac surgical procedures.

Signs of Accelerated Erythropoiesis

These signs are usually detected in patients with chronic hemolytic disease, and they appear about 5–10 days after an acute hemolytic episode. They also occur after hemorrhage and specific therapy for anemia caused by iron, folate, or vitamin B_{12} deficiency.

Laboratory Signs of Accelerated Erythropoiesis

Blood

- Reticulocytosis (Polychromatophilia, stippling)
- Macrocytosis
- Erythroblastosis
- Leukocytosis with thrombocytosis

Bone Marrow

- Erythroid hyperplasia

Ferrokinetic

- Increased plasma iron turnover
- Increased erythrocyte turnover

Biochemical

- Increased erythrocyte creatine
- Increased activity of certain erythrocyte enzymes; uroporphyrin -1-synthetase, hexokinase, glutamate oxalate transaminase

Reticulocytosis

An increased number of circulating reticulocytes continues to be the most readily and most widely used index of accelerated erythropoiesis, even though the statistical errors in enumerating them are common. The erythrocyte production rate is likely to be greatly increased when hemolysis is severe enough to produce anemia. Therefore, the reticulocyte count will probably be substantially, not minimally, increased, phenomenon that minimises the importance of the technical limitations involved in the determination.

As on date, the normal range of percentage of reticulocytes (vis-a-vis number of erythrocytes in the sample evaluated) is taken to be 0.5–1.5%, but a more accurate and realistic range is considered to be 0.8–2.5% in men and 0.8–4.1% in women.

In most varieties of hemolytic anemias the reticulocyte count consistently increases to levels that correlate fairly well with the severity of the process. Exceptions occur mainly during aplastic crisis.

Chemical Markers of Cell Age

The most promising of these difficult to ascertain chemical markers is erythrocyte creatine. Creatine levels in young cells is 6–9 times higher than those in older cells and the elevation persists for as long as 20 days of RBC lifespan, compared to the reticulocyte maturation time of 1–3 days. As a result, the erythrocyte creatine values noted after therapy of iron deficiency anemia peak later and last longer than the reticulocyte count. Erythrocyte creatine level correlates with the reticulocyte count but the relationship is not linear. The former appears to be a more sensitive indicator of red cell age than is the latter, so it may be increased in people with mild hemolytic processes when the reticulocyte count remains equivocal. Erythrocyte creatine determinations appear to be more reproducible and less affected by technician variability than in the reticulocyte count. It may provide a more accurate assessment of the intensity of the hemolytic process; however, more studies are necessary to correctly evaluate its utility.

Other Morphologic Findings in the Blood

With reticulocytosis, polychromatophilia and fine stippling are apparent on routinely stained smears of peripheral blood. Macrocytosis is found in association with most hemolytic disorders because of erythropoietin-mediated stimulation of hemoglobin synthesis and because prematurely released (shift) reticulocytes are larger than normal erythrocytes. Exceptions occur in hereditary spherocytosis and sickle cell anemia, diseases in which the intrinsic defect of the cell tends to decrease its size.

With brisk hemolysis, nucleated RBCs are released in the circulation (erythroblastosis), usually in numbers below 1% of all the nucleated cells. In infant, however, erythro-blastosis may be more striking, especially in hemolytic disease of the newborn.

Concomitantly, there may be neutrophilic leukocytosis and thrombocytosis in hemolytic anemia. These findings tend to be most common and most pronounced in patients with acute hemolytic anemias. Platelets are increased in number and also in size (giant platelets). The changes are less pronounced in chronic hemolytic processes. Leukopenia and thrombocytopenia are unusual findings in people with hemolytic disease. Their presence

suggests such diagnosis as paroxysmal nocturnal hemoglobinuria (PNH), hemolysis accompanying SLE, or, in a few cases of idiopathic autoimmune hemolytic anemia.

Bone Marrow

Erythroid hyperplasia is the hallmark in hemolytic anemia. The simplest way is to examine marrow aspirates or biopsy specimens. This way is a qualitative or semiquantitative technique, and is not suitable for precise calculation of production rates. An important fact to remember is that marrow specimens represent only a tiny miniscule sample of whole marrow and may not always accurately reflect the degree of change.

In smears of aspirated specimens, erythroid hyperplasia is manifest by a reduction in the myeloid to erythroid (M/E) precursor ratio. In hemolytic disease, the ratio is less than 1.5 and may be as low as 0.5. Because this ratio also may decrease as the result of reduction in granulocyte precursors, the meaning of the ratio must be evaluated in the light of the total clinical scenario.

Judging marrow cellularity from an aspirate is less appropriate than judging it from a marrow biopsy.

FERROKINETIC STUDIES

The plasma iron transport rate (PITR) is considered a measure of total erythropoiesis and correlates well with the degree of erythroid hyperplasia. The erythrocyte iron turnover rate (EITR) is a measure of effective erythropoiesis and correlates well with the reticulocyte production index. In hemolytic anemia, the PITR reaches 2–8 times the normal rate and EITR increases 2–4 times. Ferrokinetic measures of ineffective erythropoiesis may also increase. These determinations provide accurate information regarding rates of erythropoiesis. Nevertheless, they are unnecessary for most patients because the reticulocyte count and other easily obtained determinations are simpler, faster, much less expensive, and nearly as accurate.

LABORATORY TESTS—FINDINGS USEFUL IN DIFFERENTIAL DIAGNOSIS SPECIFIC MORPHOLOGIC ABNORMALITIES (TABLE 8.2)

A competent, careful examination of well-prepared and stained blood smear is the single most valuable tool in defining the underlying cause leading to hemolytic anemia. Detection of certain distortions of red cell shape is of particular diagnostic utility because there presence suggests only one or a few entities.

Spherocytes

Spherocytes or spherical erythrocytes are the hallmark of hereditary spherocytosis. They are also found in most cases with acquired immunohemolytic anemias, thermal injury, hypophosphatemia, or certain kinds of chemical poisoning.

Table 8.2: Morphologic abnormalities in hemolytic anemias

Cell morphology	*Description*	*Clinical disorders*
Spherocyte	Spherical; appear microcytic with no central pallor	Hereditary spherocytosis, immunohemolytic anemia, burns, chemical injury to RBC
Elliptocytes	Oval cells	Hereditary ovalocytosis, megaloblastic anemia

Contd.

Table 8.2: Morphologic abnormalities in hemolytic anemias		
Cell morphology	*Description*	*Clinical disorders*
Stomatocytes	Uniconcave red cell. Slit-like rather than circular area of central pallor	Hereditary stomatocytosis, alcoholism
Acanthocytes	5–10 spicules of various lengths irregular in spacing and thickness	Spur cell anemia with liver disease, abetalipoproteinemia
Echinocytes	10–30 spicules evenly distributed over cell surface	Pyruvate kinase deficiency, phosphoglycerate kinase deficiency, uremia
Sickle cells	Cells with sickle shape, assumed especially when deprived of oxygen	Sickle cell anemia
Target cells	Solid area in center of central pallor	Thalassemia, HbC disease, liver disease, LCAT deficiency, postsplenectomy
Schistocytes	Triangular, helmet-shaped, fragmented or greatly distorted cell small	Microangiopathic anemia, uremia, malignant hypertension, turbulent blood flow

Elliptocytes

Elliptocytes or oval cells are characteristic of hereditary elliptocytosis.

Stomatocytes

Stomatocytes, suggest a disturbance in red cell cation content, are found in association with a rare inherited hemolytic disease and also occurs as a transient abnormality in acute alcoholics.

Acanthocytes

Acanthocytes indicate disturbed erythrocyte lipid composition; they occur in association with abetalipoproteinemia and the spur cell anemia that usually accompanies hepatic cirrhosis.

Echinocytes

Echinocytes (sea urchin cells) are found in hereditary deficiencies of certain enzymes such as pyruvate kinase and phosphoglycerate kinase. They are a nonspecific morphologic abnormality and are also found in uremia.

Sickle Cells

Sickle cells are named after the unmistakable sickle-shaped poikilocytes that characterize sickle all anemia.

Target Cells

Target cells are characteristic of thalassemia, the homozygous abnormal hemoglobin states, and lecithin cholesterol acyltransferase (LCAT) deficiency; they also occur in nonhemolytic states such as obstructive jaundice and after splenectomy.

Schistocytes

Schistocytes helmet cells or other fragmented cells suggest hemolysis associated with physical trauma to the erythrocyte or with diseases affecting small blood vessels.

Erythrophagocytosis

Erythrophagocytosis, or the presence in blood of phagocytic cells containing recognizable whole red cells, is an uncommon finding. When observed, it suggests damage to the red cell

surface, especially that induced by complement-fixing antibodies, but also by protozoan and bacterial infectious agents and certain chemical poisons.

Autoagglutination

Autoagglutination may be apparent in blood smears or may even be visible to the naked eye when the blood is allowed to flow along the side of a glass container. The phenomenon is particularly observed in immunohemolytic disease caused by cold agglutinins. Autoagglutination must be differentiated from rouleaux formation, a finding observed in multiple myeloma and related diseases and the phenomenon responsible for accelerated rates of erythrocyte sedimentation.

Coombs' Test (Antiglobulin Test)

The direct Coombs test is most widely used for detection of immunohemolytic anemia. Positive tests indicate that the red cells are coated with IgG or complement components especially C3. The test is usually sufficient, but up to 5% of cases with immunohemolytic disease may have negative test results because the amount of globulin on the cell surface is below the detection limits. Rarely, patients have weakly positive test results and clinical evidence of hemolysis. Positive tests are found in up to about 35% of patients with AIDS without other evidence of immunohemolytic disease.

Osmotic Fragility Test

This test is a measure of the resistance of erythrocytes to hemolysis by osmotic stress. The test consists of exposing red cells to decreasing strengths of hypotonic saline solutions and measuring the degree of hemolysis. To construct the conventional osmotic fragility curve, percent hemolysis is plotted on the vertical axis against decreasing saline concentration on the horizontal axis. A symmetric sigmoidal curve is obtained in most individuals. Other ways of plotting curves are there. Osmotic fragility can also be described in terms of the saline concentration at which hemolysis begins (normally 0.45–0.50 g/dL) and at which it is complete (normally 0.30–0.33 g/dL). Especially useful is the median corpuscular fragility (MCF), the saline concentration at which 50% of the red cells hemolyse (normally 0.40–0.455 g/dL). Increased fragility is indicated by a shift of the curve to the left or an increased value for MCF. Osmotic resistance (reduced fragility) is signified by a rightward shift and a reduced MCF value.

Increased osmotic fragility is observed in conditions associated with spherocytosis. Hemolysis occurs in part because scant quantities of fluid need be absorbed by spherical cells to stretch their membranes to the bursting point, but other factors such as leakiness of the membrane (increased permeability to cations) and the intracellular osmotic pressure, may also be important. With prior incubation of sterile blood for 24 hours, the increased osmotic fragility of spherocytes is greatly accentuated, whereas normal cells become only slightly more fragile. The osmotic fragility of unincubated blood may be normal in some patients with hereditary spherocytosis, and incubation of the red cells may be necessary to distinguish then from normal cells. The changes in osmotic fragility with incubation time are actually quite complex but 24 hours is an optimal time for differentiating normal blood from spherocytic blood.

Increased resistance to breakdown in hypotonic saline solutions is observed in thalassemia, sickle cell anemia, and other disorders in which many leptocytes, including target cells, are found. Sometimes, a few very fragile cells may also be detectable in these cases. With prior incubation, target cells and leptocytes may even become more resistant to hemolysis, causing a further rightward shift to the curve.

Estimating osmotic fragility therefore appears to be importance mainly in confirming important morphologic finding, especially the presence of spherocytes but also that of leptocytes. A well-observed smear studied by a well-trained hematologist often provides significant information usually.

Autohemolysis

The autohemolysis test is a measure of the amount of spontaneous hemolysis that occurs after the incubation of sterile, defibrinated blood at 37°C for 48 hours. Comparison is made with values obtained when glucose or ATP is added before incubation with normal blood, minimal hemolysis (mean 2%) takes place in 48 hours and even less (0.3%) with the addition of glucose or ATP. Based on this, two types have been described: Type I-pattern is characterized by autohemolysis that is only slightly or moderately increased and is incompletely corrected by either ATP or glucose. The type II pattern is characterized by autohemolysis that is greatly increased (mean 13%) and corrected by ATP but not by glucose. A third pattern occurs in hereditary spherocytosis, namely greatly increased autohemolysis with complete or nearly complete correction with either ATP or glucose. Type I hemolysis is seen in G6PD deficiency and type II in pyruvate kinase deficiency. However, the patterns are neither sensitive nor specific. The investigation is only of historical relevance today.

Tests for Hemolytic Disorders Associated with Heinz Body Formation

In certain disorders, the hemolytic process depends on precipitation of hemoglobin, with the formation of inclusions known as Heinz bodies. These inclusions are rapidly eleminated by spleen. Heinz body formation is the principal mechanism of hemolysis in G6PD deficiency and related disorders, in unstable hemoglobin disease, in the thalassemias, and in certain kinds of chemical injury. These bodies are observed only with supravital staining techniques. Cells containing these inclusions may be found in the blood during an acute drug reaction in G6PD deficient individuals and also in splenectomized patients with unstable hemoglobin disease. With an intact spleen they are hardly ever seen.

Inducing Heinz body formation *in vitro* is possible by incubating RBCs with acetylphenylhydrazine. The number, size and rate of formation of the inclusions tend to be greater in cells from patients with the disorders mentioned previously than in other individuals, however, the frequency of occurrence of false-positive result in association with many anemias has limited the utility of this procedure.

The glutathione stability test depends on the observation that when sensitized cells are exposed to acetylphenylhydrazine, the glutathione level falls rapidly to low levels; the rate and amount of decrease exceed those of normal cells. Investigation is technically difficult and hardly ever used.

The ascorbate-cyanide test is a sensitive measure of abnormal susceptibility of hemoglobin to peroxidative denaturation. It depends on the coupled oxidation of ascorbate with oxyhemoglobin to produce hydrogen peroxide. When catalase in inhibited with cyanide, the glutathione-dependent system is required to protect against peroxidative damage to hemoglobin. The test is easy and fast to perform and results are strongly positive in patients with deficiencies of G6PD or glutathione peroxidase and with some unstable hemoglobin disorders. Lesser degrees of positivity have been reported in association with deficiences of glutathione reductase or pyruvate kinase and in individuals heterozygous for G6PD deficiency.

For simplicity, only the ascorbate–cyanide test is preferable for routine use. More specific tests, such as enzyme spot tests or the heat stability test for unstable hemoglobins, also are useful in differential diagnosis of these disorders.

APPROACH TO DIAGNOSIS

Diagnosis here is a double-step process: first, demonstrating that a hemolytic anemia exists and, second determining the specific cause of the condition. A usual error made in practice is the pursuit of the second objective before the first is established. Reversal of this process can wasteful and unproductive.

Does Hemolytic Anemia Exist?

Hemolytic anemia is usually suspected when anemia exists in conjunction with signs of increased RBC destruction and/or signs of accelerated RBC production. However, no single test for hemolytic anemia exists that is absolutely reliable. Therefore, the diagnosis necessitates very careful analysis of erythrokinetic information along with due consideration of situation that might be misleading signs of accelerated erythropoiesis characteristically appear after hemorrhage, the intentional or inadvertent treatment of certain nutritional deficiencies, or the recovery from marrow failure. Apparent evidence of an increased rate of RBCs destruction is often noted in conditions associated with ineffective erythropoiesis, disorders of bilirubin catabolism and loss of blood into a tissue space. If these situations can be excluded, the signs become reliable indicators of hemolytic disease.

The findings characteristic of hemolytic anemia of recent onset differ considerably from those typically noted in association with established chronic disease. In the latter, the compensatory increases in the rate of RBC production have been implemented, and signs thereof are prominent. Thus, the most common manifestation of chronic hemolytic anemia and reticulocytosis, is often associated with various signs of excessive blood destruction. In contrast, the onset of acute hemolytic anemia is not accompanied by signs of accelerated red cell production. Clinicians may suspect such a diagnosis because of the abrupt onset of hemoglobinuria or other signs of intravascular hemolysis or because of a rapid fall in blood hemoglobin concentration from previously stable levels. As a rule, a decrease of more than 1.1 g/dL/week is indicative of hemolysis, hemorrhage, or hemodilution. If hemorrhage and hemodilution can be excluded, the presence of hemolytic anemia is established.

CONDITIONS THAT CAN SOMETIMES BE MISTAKEN FOR HEMOLYTIC ANEMIA

Associated with anemia and reticulocytosis

- Hemorrhage
- Recovery from iron, folate or vitamin B_{12} deficiency
- Recovery from marrow failure, as by cessation of alcohol consumption or stoppage of cytotoxic druges

Associated with anemia and acholuric jaundice

- Ineffective erythropoiesis (intramedullary hemolysis)
- Loss of blood into a body cavity.

Acholuric jaundice without anemia

Marrow invasion (myelofibrosis, metastatic disease) myoglobinuria.

The anemias associated with acute hemorrhage and those with partially treated deficiency states are characterized by transient anemia and reticulocytosis. They can

usually be distinguished from hemolytic disease by the absence of icterus and by a rising VPRC on subsequent determinations.

Anemias caused by ineffective eythropoiesis often are accompanied by acholuric jaundice and by erythroid hyperplasia of the marrow; however, the reticulocyte count is often not raised. In equivocal situations, a necessary step may be to measure RBC survival which is distinctly shortened in hemolytic disease and normal or nearly normal in association with ineffective erythropoiesis. The two conditions can also be differentiated on the basis of results obtained from ferrokinetic studies.

An especially confusing situation may arise after occult hemorrhage into the retroperitoneal space or other tissue compartments; anemia develops rapidly and reticulocytosis follows. Furthermore, indirect hyperbilirubinemia may occur as the result of reabsorption of the products of hemoglobin breakdown at the site of hemorrhage. Thus, the picture of hemolytic anemia is simulated in several ways. Diagnosis depends on detecting signs of the hemorrhage itself or of the disease process leading to it. If occult hemorrhage is suspected, serial observations usually clarify the situation; once the hemorrhage stops, the VPRC, reticulocyte count, and bilirubin values return to normal.

In patients with acholuric jaundice but without anemia, the differential diagnosis lies between a compensated hemolytic state and Gilbert's syndrome or other disorders of bilirubin metabolism. Reticulocytosis or morphologic erythrocytic abnormalities are typical findings in the former. In mild compensated hemolytic disease, however, one cannot always be certain that the hematologic values will be abnormal. Fasting induces an exaggerated increase in bilirubin in patients with Gilbert's syndrome, and this plenomenon may be useful in distinguishing Gilbert's syndrome from hemolytic anemia. Measurement of RBC survival provides more assurance; however, if the patient is asymptomatic, little may be gained from precise diagnosis.

Anemia associated with marrow invasion may be accompanied by erythroblastosis and bizzare abnormalities of RBC morphology. Mild reticulocytosis may develop because of premature release (shift to the left). Usually, patients do not have jaundice, however, and evidence of invasive disease may be detected by examination of the bone marrow.

Specific Cause Determination

The diagnostic analysis should begin with information form the history taking and the results of blood smear examination and an antiglobulin test. From data so obtained, five groups of patients can be distinguished.

1. Those in whom the diagnosis is clear because of obvious exposure to infections, chemical, or physical agents.
2. Those with antiglobulin-positive hemolytic anemia, such individuals may be presumed to have immunohemolytic anemia. The underlying disease as well as a serologic study of the nature of the antibody must be undertaken.
3. Those with antiglobulin-negative, spherocytic hemolytic anemia. Such patients are probably cases of hemolytic anemia. Confirm this by osmotic fragility test and also study the familial involvement. Some unusual causes of spherocytosis without any familial involvement may warrant consideration, immunohemolytic anemia may be associated with spherocytosis and is occasionally associated with negative antiglobulin test. Procedures more sensitive than the routine Coombs' test may be useful in such circumstances. Exposure to chemical or infectious agents producing spherocytosis may not always be easy to establish.

4. Those with other specific morphological abnormalities of erythrocytes. The significance of various types of poikilocytes has been discussed earlier. Some poikilocytes, such as elliptocytes and sickle cells, are virtually pathognomonic findings.
5. Those with no specific erythrocytic morphological anomalies and a negative reaction to antiglobulin test. These patients warrant a battery of screening tests, including hemoglobin electrophoresis, the heat denaturation test for unstable hemoglobin disease, the ascorbatecyanide test, spot test for deficiencies of pyruvate kinase and G6PD, and a screening test for paroxysmal nocturnal hemoglobinuria.

If all of these procedures yield normal results, making the diagnosis is likely to be difficult. One of the rarer erythrocyte enzyme deficiencies is possible, but these entities can only be established only by employing specific assays.

TREATMENT

Precise management is dependent upon the diagnosis. Ascertain the cause of anemia and treat it if possible. Further exposure to any chemical, drug, or any other etiologic agent must stop, or if a bacterial agent is responsible, a specific antibacterial therapy must be instituted. Appropriate treatment of patients with symptomatic hemolytic anemias is determined with consideration of the underlying disease.

A patient with an acute attack of hemolysis should be treated by appropriate measures to relieve shock, if present, to maintain fluid balance, and to allow renal repair if the kidneys have been damaged. Blood transfusions, so useful in acute anemia of other types, must be used with caution in the treatment of patients with acquired hemolytic because even with careful blood matching, destruction of the transfused blood with an increase in the burden on the excretory organs and sometimes with ensuing thrombosis that may occur. Nevertheless, when blood destruction is rapid, the dangerous consequences of shock can be met only by administration of adequate amounts of blood. The presence of antibodies makes the blood matching that much more difficult. Whenever possible, the patients, blood should be genotyped before the first transfusion, with determination of the specific antibodies. Blood homologous as to ABO and Rh blood groups should be matched with that of the patient and the best match should be used. Samples of the recipient's serum and prospective donor's cells should be incubated together in a test tube at 37°C for 1 hour to ascertain whether the cell will undergo hemolysis by the hemolysins contained in the serum. Despite all such precautions, transfusion may nevertheless accelerate hemolysis. Such patients may require exchange transfusion.

Mildy damaged red cells are removed by spleen and severely damaged red cells are destroyed by macrophages in many organs, especially the liver. Therefore, patients with diseases associated with mild red cell defects are most likely to respond to splenectomy. The best case to respond is hereditary spherocytosis. Hereditary elliptocytosis also responds well. Anemia associated with certain of the red cell enzymatic deficiencies may lessen after splenectomy, but the response is only partial, and continuing signs of hemolysis are to be expected; such responses have been observed in patients with Deficiencies of pyruvate kinase, hexokinase or glucose-phosphate isomerase. In mild-to-moderate case of unstable hemoglobin disease, splenectomy helps.

In individuals with immunohemolytic anemia, splenectomy may be effective not only because the spleen is a site of RBC destruction, but also because it may be a source of antibody production. In general, patients with warm antibodies are more likely to respond than those with cold antibodies, patients with splenomegaly respond better, and those with

incomplete antibodies, especially if present in small amounts, are more likely to respond favorably to splenectomy than those with complete agglutinins or complement-fixing antibodies. In spite of these generalizations, the results, of splenectomy are still quite unpredictable, which has led to efforts to develop methods for assessing the erythroclastic function of the spleen in the individual patient. Such methods are based upon the injection of ^{51}Cr-labeled erythrocytes and subsequent monitoring of the radioactivity over the spleen and liver with a unidirectional scintillation counter. Excessive splenic sequestration suggests that the spleen is playing a major role in hemolysis, and that its removal may be beneficial.

Steroid hormones are of tremendous value in the therapy of patients with certain forms of immunohemolytic anemia. For patients not responding to steroids, immunosuppression may be considered.

For some patients with chronic hemolytic disorders, folic acid is helpful in preventing megaloblastic crisis. A dose of 0.15–0.3 mg/day is ample for this purpose. Such therapy is particularly valuable for patients with severe continuing hemolysis; for people subsisting on marginal diets; and for pregnant patients.

RECAPITULATION IN BRIEF

Evidences of Hemolysis

I. Increased breakdown of hemoglobin

- Jaundice and hyperbilirubinemia (hemoglobin breakdown and conversion to bilirubin)
- Reduced plasma haptoglobin and hemopexin (free hemoglobin combines with both of them)
- Increased plasma LDH (released from breaking red cells)
- Increased urinary urobilinogen (excessive bilirubin pool)
- Increased fecal urobilinogen (Increased bilirubin pool)
- Hemoglobinuria, and hemoglobinemia
- Methemalbuminemia

] Evidence of intravascular hemolysis

- Hemosiderinuria

II. Compensatory erythroid hyperplasia

- Reticulocytosis, erythroblastemia (erythroid cells overactivity, hence release of premature cells)
- Macrocytosis and polychromasia (premature cells in circulation)
- Erythroid hyperplasia of bone marrow (reversal of M:E ratio) (Compensatory mechanism)
- Skeletal X-ray (Widening of marrow space)
- Radiological changes in skull and tubular bones (in congenital hemolytic anemia only)

III. Damage of red cells

- Spherocytosis, increased in skull and tubular bones
- Fragmentation of red cells
- Heinz bodies

IV. Demonstration of shortened life span of red cells

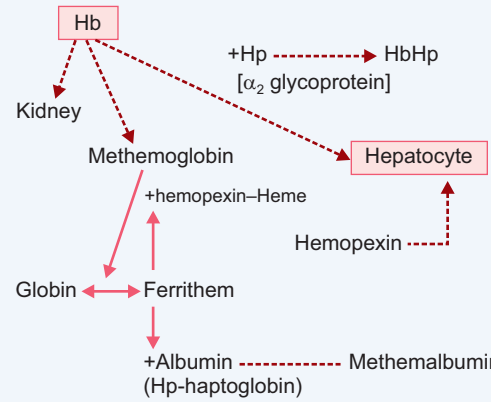
Catabolism of Circulating Hemoglobin

- See Flowchart 8.1.

Hemolytic Jaundice

- Is acholuric
- There is no pruritus or bradycardia
- Complications
- Gallstones
- Liver damage.

Flowchart 8.1



Haptoglobin (HP)

- Normal plasma HP = 1–1.5 g/L
- Levels assessed by a rapid latex agglutination test
- Levels reduced in Intra/extravascular hemolysis
- Hb (a b dimers). One HP molecule binds 2 dimers which are removed from plasma by RE system (liver).
- Levels under 1 g/L imply × 2–3 times hemolysis or the half-life of red cells is 17 days or less.
- Decreased HP levels occur in
  - Hepatocellular disease
  - Hereditary disorders
- Increased HP levels occur in some acute and chronic liver diseases.

Hemopexin

- Normal plasma level = 0.5–1 g/L
- Is a β glycoprotein and binds heme in 1: 1 ratio
- Is manufactured by liver, it does not bind Hb
- The ferrithem hemopexin is removed by liver
- Its concentration is measured by radial immunodiffusion method
- In most intravascular hemolysis, its levels are diminished

Plasma LDH

- Not as high as in megaloblastic anemia
- Isoenzyme that rises in hemolytic anemia is different.

INTRAVASCULAR HEMOLYSIS EVIDENCES

- Renal tubular cells convert globin into amino acids which are returned to body stores
- Renal tubular cells convert heme into bilirubin (renal bilirubin formation is the major site of increased bile pigment formation in hemolytic anemia).
- Methemalbumin is the last heme pigment to leave the body.

Hemoglobinemia, Hemoglobinuria

Causes of hemoglobinuria

Acute hemoglobinuria

- Incompatible blood transfusion
- Hemolytic anemia due to drugs and chemicals
- Favism
- Paroxysmal cold hemoglobinuria
- March (exertional) hemoglobinuria
- Hemolytic anemia due to infections (mainly *Clostridium welchii*)
- Blackwater fever
- Hemolytic anemia associated with eclampsia
- Hemolytic–uremic syndrome
- Hemolytic anemia due to burns
- Snake and spider bites

Chronic hemoglobinuria

- Paroxysmal nocturnal hemoglobinuria
- Cardiac hemolytic anemia
- Cold hemagglutinin disease.

Normal free plasma Hb = 0.6 mg%

When Hp binding capacity is exceeded free Hb in plasma may rise to 100–200 mg% (plasma then appears pink or red)

Methemalbumin imparts brown tinge to plasma (It is identified chemically by Schumm's test)

Hemosiderinuria

- Particularly seen in chronic hemolytic anemia, especially in PNH
- The exfoliated cells in urine give a positive Perl's reaction
- Transient hemosiderinuria may occur in acute hemolytic anemia.

Urobilinogen

- Normal fecal urobilinogen excretion 50–300 mg/day
- Normal urinary urobilinogen excretion <4 mg/day (Qualitative estimation done by Ehrlich's test)
- Urinary urobilinogen on standing darkens (urobilin)

Erythroid Hyperplasia

- Reticulocyte count rises in hemolytic anemia (5–20%)
- Reticulocyte count may become zero (in aplastic crises of hereditary spherocytosis).
- Polychromatophils are 30% larger than reticulocytes
- Bone marrow hyperplasia is usually normo-blastic but may, sometimes, be megaloblastic
- Skeletal changes
- Skull: Ground glass appearance of outer table, hair on end appearance (persist postpuberty)
- Tubular bones: Cortical thinning, increase in transverse diameter (disappear at puberty)
- Osteosclerosis

Red Cell Damage

Spherocytosis

- Hereditary
- Acquired
- Autoimmune hemolytic anemia
- Hemolytic anemia
  - Chemicals
  - Burns
  - Infections

RBC osmotic fragility: Increases in hemolytic anemia
Normal values = 0.40–0.45% saline (mean corpuscular fragility)
It is a sensitive test for spherocytosis
Target cells have diminished osmotic fragility

HEMOLYTIC ANEMIA

Fragmentation of RBCs occurs especially in

- Hemolytic anemia due to chemicals
- Cardiac hemolytic anemia
- Microanglopathic hemolytic anemia

Heinz bodies

- Made of denatured globin
- Demonstrated by supravital staining
- Are formed when reducing power of red cells cannot counter the excessive oxidative stress
- Occur in
  - Direct action of oxidant drugs
  - G6PD deficiency
  - Unstable Hbs
  - α and β thalassemias
  - Post-splenectomy

Shortened Red Cell Life Span

Can be assessed by using

- 51Cr (chromium)
- DF32P(phospho-fluoridate)
- 3H-DFP

The radioisotope labeled RBCs are infused and radioactivity is measured over liver and spleen and the results are expressed as counts over spleen and liver in excess of those expected. If count is maximum over spleen, splenectomy will help the patient. If on both liver and spleen, splenectomy will help partially.

PREDOMINANT RED CELL MORPHOLOGY COMMONLY ASSOCIATED WITH NONIMMUNE HEMOLYTIC DISORDERS (FIG. 8.1)

Spherocytes

- Hereditary spherocytosis
- Acute oxidant injury (HMP shunt defects during hemolytic crisis, oxidant drugs, and chemicals).
- Clostridium welchii septicemia
- Severe burns, other cell thermal injuries
- Spider, bee and snake venoms
- Severe hypophosphatemia
- The features shown in the figure are not necessarily seen in every hemolytic anemia.

Bizarre Poikilocytes

- Red cell fragmentation syndromes (microan-giopathic and macroangiopathic hemolytic anemias)
- Hereditary elliptocytosis in neonates
- Hereditary pyropoikilocytosis

Elliptocytosis

- Hereditary elliptocytosis
- Thalassaemias

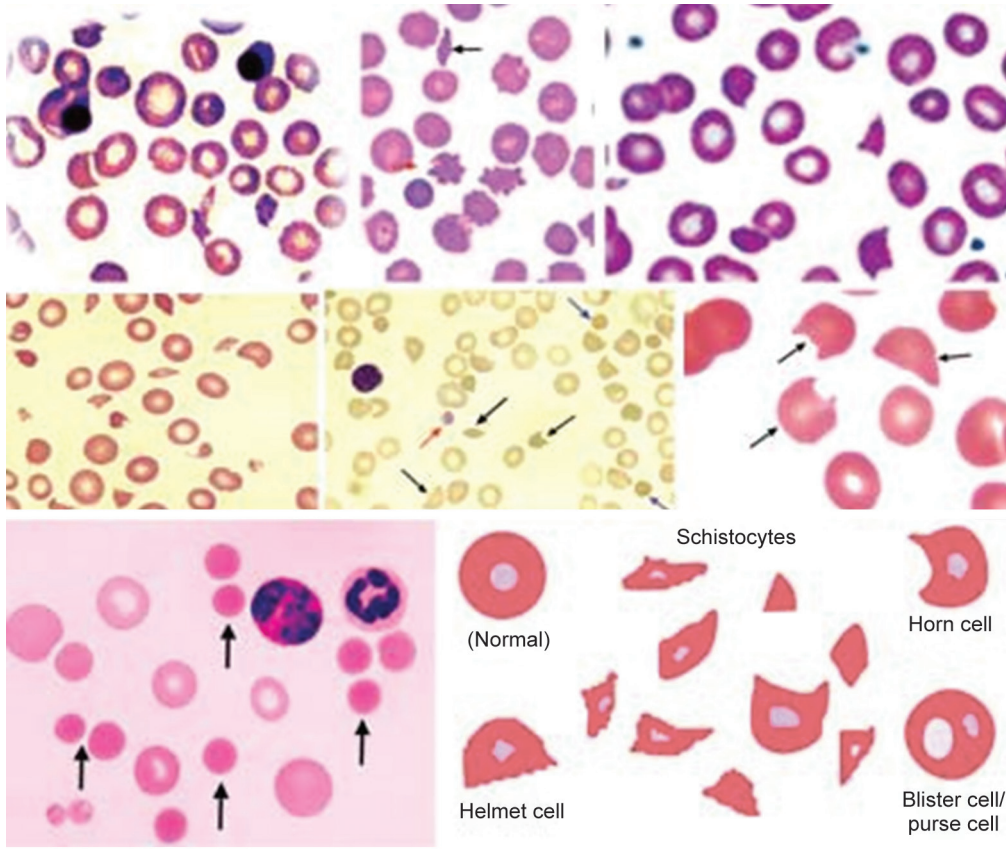


Fig. 8.1: Peripheral smear presentations in hemolytic anemias

Stomatocytes

- Hereditary stomatocytosis and related disorders
- Stomatocytic elliptocytosis

Irreversibly Sickled Cells

- Sickle cell anemia
- Symptomatic sickle syndromes

Intraerythrocytic Parasites

- Malaria
- Babesiosis
- Bartonellosis

Prominent Basophilic Stippling

- Thalassemias
- Unstable hemoglobins
- Lead poisoning
- Pyrimidine-5-nucleotidase deficiency

Spiculated or Crenated Red Cells

- Acute hepatic necrosis (spur cell anemia)
- Uremia
- Infantile pyknocytosis
- Abetalipoproteinemia
- McLeod blood group

Target Cells

- Hemoglobins S, C, D and E
- Thalassemias
- Hereditary xerocytosis.

Nonspecific or Normal Morphology

- Embden–Meyerhof pathway defects
- HMP shunt defects
- Adenosine deaminase hyperactivity with low red cell ATP
- Unstable hemoglobins
- Paroxysmal nocturnal hemoglobinuria
- Dyserythropoietic anemias
- Copper toxicity (Wilson's disease)
- Cation permeability defects
- Erythropoietic porphyria
- Vitamin E deficiency
- Hypersplenism

HEREDITARY SPHEROCYTOSIS AND OTHER ANEMIAS DUE TO ABNORMALITIES OF THE ERYTHROCYTE MEMBRANE

RED CELL MEMBRANE: GENERAL CONSIDERATIONS

Red cell membrane structure is a bilayer of phospholipids intercalated with molecules of unesterified cholesterol and glycolipids. The phospholipids are asymmetrically organized, with the choline phospholipids (phosphatidyl choline and sphingomyelin) primarily on the outside of the bilayer and the amino phospholipids (phosphatidyl ethnolamine and phosphatidyl serine) on the inside. The membrane also contains proteins which are asymmetrically organized. All glycoproteins are exposed on the outer membrane surface,

and these are red cell antigens and receptors (such as glycophorins) or transport protein (that is, the anion transport channel). These integral membrane proteins penetrate or span the lipid bilayer, interact with the hydrophobic lipid core, and are tightly bound to the membrane. A separate protein network forms the memebrane cytoskeleton which interacts with both the integral membrane proteins and the lipid bilayer. The cytoskeleton is composed of spectrin, ankyrin, protein 4.1, pallidin (protein 4.2) and actin. Spectrin is made up of αβ heterodimers which, at the head end, interact with other spectin α β dimers to form heterotetramers $(\alpha\beta)_2$ in which the N-terminal end of α-spectrin and the C-terminals of β-spectrin form the head region of the hetero-dimer. These spectrin tetramers are the major structural subunits of the membrane skeleton. The spectrin dimer-tetramer interconversion is governed by a simple thermodynamic equili-brium that favors spectrin tetramer formation. At the distal end, spectrin tetramers interact with protein 3 (the anion exchange channel) via ankyrin, and this interaction is most important for attachment of the cytoskeleton to the membrane. Also at the distal end, spectrin tetramers interact with actin via protein 4.1. In addition there are other membrane protein interactions not involving spectrin. These interactions include glycophorin C to protein 4.1, and pallidin (protein 4.2) to band 3; the later interaction is thought to stabilize ankyrin in the membrane. The horizontal protein interactions, mainly the interactions of spectrin dimers, are responsible for the lateral deformeability of the cytoskeleton. Thus these protein interactions are responsible for the overall stability of the membrane, thereby allowing the red blood cell to be deformable during its lifespan within the circulation.

Hereditary RBC membrane disorder result from alterations in the quantity and/or quality of these individual proteins and their interactions with each other. Hereditary spherocytosis is caused by an uncoupling of the cytoskeleton from the lipid bilayer, thereby leading to membrane instability with loss of lipids and some integral membrane protein. Hereditary elliptocytosis disorders are the result of defects in spectrin dimer interactions, thereby leading to disruption of the skeletal lattice and RBC fragmentation.

HEREDITARY SPHEROCYTOSIS (HS) (FIG. 8.2)

Hereditary spherocytosis (HS) is a familial hemolytic disorder characterized by anemia, intermittent jaundice, splenomegaly, and responsiveness to splenectomy. Clinically these cases may range from very mild asymptomatic to fulminant hemolytic anemias. The morphologic hallmark of HS is the microspherocyte, which is caused by loss of membrane surface area, and is characterized by an abnormal osmotic fragibility *in vitro*. The intrinsic genetic defect causes qualitative and quantitative defects in membrane proteins; and these, in turn, are the result of a variety of molecular defects that are now being defined.

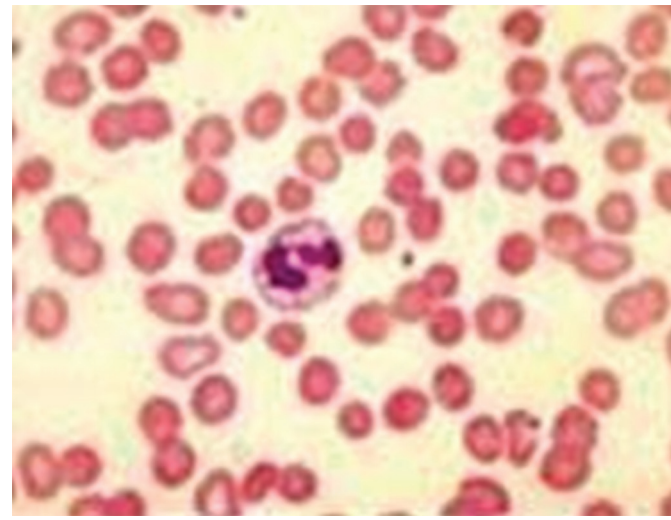


Fig. 8.2: Hereditary spherocytosis, peripheral smear

HS is transmitted as an autosomal dominant trait and the

identification of the disorder in multiple generations of affected families is the rule. Homozygosity for the dominantly transmitted HS gene has not been identified, thus suggesting that the homozygous state is incompatible with life. Nearly 25% of all newly diagnosed patients do not demonstrate dominant inheritance pattern, and the parents of these patients are clinically and hematologically normal. New mutations have been implicated that may explain some of these sporadic cases; in support of this is the fact that approximately 50% of the offspring of individuals with sporadic HS also have the disease. An autosomal recessive mode of inheritance also occurs and this is supported by the descriptions of families in which apparently normal parents have had more than one affected child. This recessive pattern may account for 20 to 25 percent of all HS cases. It is manifested only in homozygous or compound heterozygous individuals and often is associated with severe hemolytic anemia.

Pathogenesis (Flowchart 8.2)

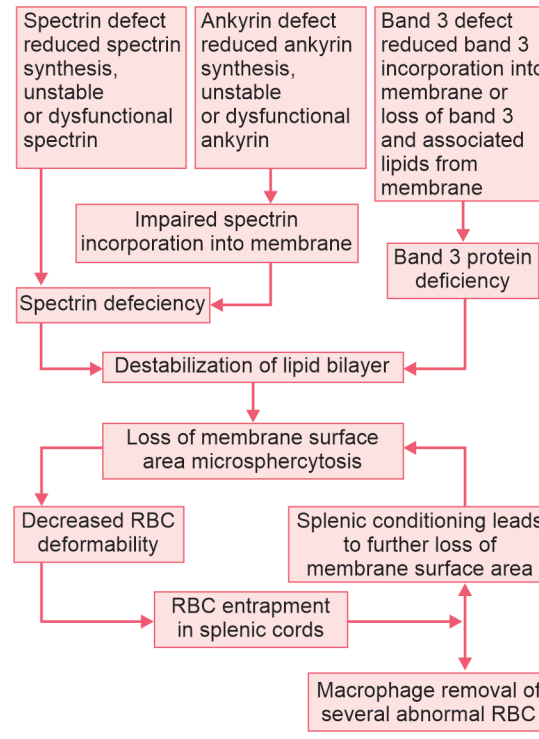
In hereditary spherocytosis, hemolysis, results from the interplay of an intact spleen and an intrinsic membrane protein defect which leads to an abnormal red blood cell.

The first biochemical defect recognised in patients with HS was *spectrin deficiency* and the degree of spectrin deficiency was reported to correlate with the extent of spherocytosis, the degree of the osmotic fragility, and severity of hemolysis. In some cases spectrin deficiency is the result of impaired synthesis, whereas in other instances it is caused by qualitative or quantitive deficiencies of other proteins which integrate spectrin into the cell membrane. In the absence of these binding proteins, free spectrin is degraded, thereby leading to spectrin deficiency. Mutations of β-spectrin are associated with recessive forms of HS whereas mutations of β-spectrin occur in families with autosomal dominant forms of HS.

Ankyrin is the principal binding site for spectrin on the red cell membrane, and studies of cytoskeletal protein assembly in reticulocytes indicate that ankyrin deficieny leads to decreased incorporation of spectrin on the membrane. Thus in cases of HS caused by ankyrin deficiency there is a proportional decrease in spectrin content although spectrin synthesis is normal. A 75–80% patients with autosomal dominant HS have been found to have combined spectrin and ankyrin deficiency and that the two proteins are diminished equally.

Band 3 deficiency has been recognized is 10–20% of patients with mild to moderate autosomal dominant HS. These patients have 20–40% decrease is band 3 content. They also have a proportionate decrease in protein 4.2 content on the membrane. In HS subjects deficient is band 3, the deficiency is considerably greater in older RBC, thus suggesting that band 3 protein is unstable because of poor binding or a defective assembly of band 3 on the membrane.

Flowchart 8.2: Pathophysiology of HS


Spectrin defect reduced spectrin synthesis, unstable or dysfunctional spectrin
Ankyrin defect reduced ankyrin synthesis, unstable or dysfunctional ankyrin
Band 3 defect reduced band 3 incorporation into membrane or loss of band 3 and associated lipids from membrane
Impaired spectrin incorporation into membrane
Band 3 protein deficiency
Spectrin defeciency
Destabilization of lipid bilayer
Loss of membrane surface area microsphercytosis
Decreased RBC deformability
Splenic conditioning leads to further loss of membrane surface area
RBC entrapment in splenic cords
Macrophage removal of several abnormal RBC

Pallidin (protein 4.2) deficiency (complete or almost complete) has been described in some cases of autosomal recessive hereditary hemolytic animia. The phenotypes described in these reports have been quite heterogenous, with RBC morphology characterized by spherocytes, elliptocytes, is sphero-ovalocytes. Deficiency of protein 4.2 in HS is relatively common in Japan.

Erythrocyte Abnormalities

Loss of membrane surface area is the major pathologic feature of HS RBCs. Membrane loss transforms red cells from biconcave disks to spherocytic forms, this reduces their deformability. Consequently, HS red cells have limited ability to pass through the small fenestrations in the venous sinuses of the spleen. The primary cytoskeleton defect in HS results in a variety of secondary metabolic changes. These metabolic effects include increased sodium and potassium flux across the HS membrane, which leads to increased membrane sodium-potassium ATPase activation; accelerated ATP breakdown; increased rate of glycolysis; and a decreased concentration of 2, 3 diphosphoglycerate (2, 3-DPG). The latter is probably a consequence of a lower intracellular pH. However, because the decrease in red cell 2, 3 DPG and intracellular acidosis both are corrected by splenectomy they have been attributed to detention of spherocytes within the spleen. HS red cells are slightly dehydrated although the reason for diminished RBC water content is not known.

Role of the Spleen in the Pathophysiology

The survival of infused HS red cells into normal recipients is reduced, whereas the survival of normal red cells in HS subject is normal. This basic observation demonstrates an intrinsic defect in HS red cells. A second fundamental observation is that the survival of HS red cells in splenectomized recipient is nearly normal. Moreover, despite the persistence of spherocytosis following splenectomy, red cell survival is normal. Combined together, these observations demonstrate than an intrinsic red cell defect leads to RBC destruction, but only in the presence of an extrinsic factor, an intact spleen.

Splenic entrapment: Radiolabeled spherocytes are sequestered selectively in the spleen; this happens because of the unique anatomy of the splenic vasculature. Arterial blood enters directly into the splenic cords, a network of channels formed by reticulum cells and lined by macrophages. Most of the blood that enters the splenic cords passes rapidly through direct channels which re-enter the venous system after traversing fenestrations between the lining endothelial cells. A minor fraction of blood in the splenic cords percolates more slowly through this maze before reaching the venous sinuses. The hematocrit of blood from the splenic cords is high, the microevironment is acidic, and the red cells are exposed to macrophages which line these channels. The size of fenestrations in the venous sinuses is small relative to the RBC size, and to pass through requires some degree of deformability of the RBC membrane. This however, is a major problem for HS red cells which have lost surface area and are dehydrated. Examination of spleens removed from HS patients reveal congested cords and relatively empty venous sinuses. The most severely damaged spherocytes, cells unable to negotiate the fenestrations in the venous sinus are removed from the circulation by macrophages. However, impaired deformability of HS red cells is only significant for passage of these cells through the spleen. Following splenectomy red cell survival is normal, even through spherocytes persist and sometimes are increased.

Splenic conditioning: Besides trapping HS red cells the spleen also "conditions" these cells in a way that accelerates membrane loss and spherocyte formation. Some conditioned

red cells reenter the systemic circulation, demonstrated most convincingly with Fe^{59} labelling of HS cells. Cohorts of cells so labeled gradually shift from osmotically normal to osmotically fragile cells during their circulation in patients with intact spleens. Osmotically fragile microspherocytes are concentrated in and emanate from the splenic pulp. The more conditioned or spheroidal cells are responsible for the most fragile portion of the fresh osmotic fragility. The mechanism of splenic conditioning is not clear. It may be the result of hemoconcentration and erythrostasis with macrophage inflicted membrane injury. It may be related to the lower pH in the spleen as well as sequestered red cells. This pH change may activate KCl cotransport, leading to cell dehydration, and further decreased deformability. Because the calculated transit time for spherocytes is short relative to the time required for severe metabolic compromise it is unlikely that metabolic depletion is important, and the ATP content of HS cells in spleen is normal. The conditioning effect of the spleen is a cumulative injury, thought to result from several passages through this organ.

Clinical Features

Anemia, jaundice, and splenomegaly are the clinical features of HS most commonly encountered. However, signs and symptoms are highly variable, both with respect to age of onset and severity. For example anemia and hyperbilirubinemia may be so high as to require exchange transfusion in the neonatal period. Conversely, cases have been reported where initial anemic problem was first recogniszd at age 75 years. Anemia usually is mild to moderate, but sometimes it is very severe, and in some pedigrees, there may be no anemia. Jaundice is likely to be most prominent in the newborn. About 30–50% of adult with HS have a history of jaundice during the first week of life. The magnitude of hyperbilirubinemia may be such as to require exchange transfusion. Beyond the neonatal period, jaundice is rarely intense.

Typically, icterus is intermittent and is associated with fatigue, cold exposure, emotional stress or pregnancy. An increase in scleral icterus and a darker urine color is commonly seen in children with nonspecific viral infection. Even when patients have no visually detectable jaundice there usually is laboratory evidence of ongoing hemolysis. Splenomegaly is the rule. No apparent correlation exists between spleen size and disease severity. The liver is normal in size and function.

Clinically, HS is classified as mild, moderate, and severe. *Moderate HS* is the most common presentation, recognised as a chronic hemolytic disorder with characteristic microspherocytes on the peripheral smear. Usually, the pattern of transmission is autosomal dominant, although recessive inheritance is also seen. *Moderate HS* accounts for 60–75% of all cases. *Mild HS* occurs in 20–30% of cases of autosomal dominant HS. In these patients anemia is not generally present because the bone marrow is able to fully dish out RBCs at the same rate as they are lost, and there is little or no splenomegaly. As this group of patients usually are asymptomatic, they are often undiagnosed till later in life. Sometimes they are caught as a result of hemolytic or aplastic episodes triggered by an infection.

Severe HS occurs in about 5% of all HS cases. It is characterized by severe hemolytic anamia, requiring red cell transfusions, and usually having an incomplete response to splenectomy. Here, the pattern of inheritance is almost always recessive. The silent carrier of HS exists in families with autosomal recessive HS. In most of these cases, the parents of an affected patient have no sign of HS or only a mild increase in the reticulocyte count, a few spherocytes on peripheral smear, a minimally abnormal incubated osmotic fragility or an abnormal spectrin content detected when using sensitive electrophoretic techniques.

The two important complications seen in HS are episodes of worsening anemia and the development of bilirubinate gallstones. Exacerbations of anemia occur in almost all HS cases, even in mild category ones. For instance, a previously mild anemia can become much more severe during pregnancy, usually because of raised plasma volume, but occasionally a result of enhanced hemolysis. As with other chronic hemolytic states, some anemia crises are preceded by a febrile illness and may be observed in more than one member of a family. In some cases there may be increased hemolysis (decreased Hb, increased reticulocytes and raised bilirubin concentration) associated with nonspecific fevers. In other cases, just as with other cases with chronic hemolysis, hypoplastic crises resulting from human parvovirus infection are seen. These transient aplastic crises are characterized by an arrest of erythropoiesis leading to a sudden decrease in hemoglobin concentration and reticulocytopenia. Recovery occurs in 7–10 days being heralded by reticulocytosis and thrombocytosis, sometimes, enhanced anemia is the result of exhaustion of folate reserves by sustained increase in net DNA synthesis. Megaloblastic arrest of erythropoiesis has been observed most commonly during pregnancy as in association with hepatic disease. Cholelithiasis is common in HS just as in other chronic hemolytic disorders. Pigment type of gallstones may be found in very young children, but its incidence increases markedly with rising age and are seen in 40–80% of adult HS cases. The history of family members with cholelithiasis in the 2nd or 3rd decade of life in a clue to the possibility of HS or other chronic hemolytic disorder. In mild HS cases, cholelithiasis may be the first presenting sign of an underlying red cell disorder.

Other features seen are—heterotopia of the bone marrow rarely in the renal pelvis or along the vertebral column (often mistaken for tumors). After splenectomy they undergo fatty metamorphosis. Hemosiderosis and multiple endocrine disorder resulting from transfusion-induced iron overload have been described. Symptomatic iron overload has also been reported in a few non-transfused HS cases who are heterozygous for the hemochromatosis gene. Chronic leg ulcers are an unusual complication in adults with HS.

Laboratory Findings

The typical laboratory features of HS include minimal or no anemia, reticulocytosis, an increased MCHC, spherocytes an peripheral blood smear hyperbilirubinemia and abnormal osmotic fragility test (Fig. 8.2).

Although *reticulocytosis* with values ranging from 5–20% is common, some mild HS cases come to medical attention during a hypoplastic anemic crises, and in these situations the initial reticulocyte count can be extremely low. Usually, however, these patients have hyperbilirubinemia as evidence of accelerated RBC destruction. The exact reason for the marked reticulocytosis seen in mild to moderate HS in minimal anemia is ill-understood.

A raised *MCHC* (mean corpuscular hemoglobin concentration) is a characteristic finding in HS. MCHC values more than the upper limit of normal (35–36%) are common. This increased MCHC is a result of mild cellular dehydration. The mean corpuscular volume (MCV) usually is normal. However, keeping in mind the degree of reticulocytosis, the MCV in HS patients actually is low. This relatively low MCV may reflect membrane loss and cell dehydration. Fluctuations of MCH parallel those of the MCV.

RBC morphology, though distinctive, is not diagnostic varying degrees of polychromatophilia and anisocytosis are noted. Anisocytosis is prominent, and the smaller cells are

spherocytes, recognized by the intensity of staining, lack of central pallor and size. Because the degree of spherocytosis or "conditioning" is a function of cell age, reticulocytes and young red cells are morphologically normal. In mild HS up to 1–3, and in moderate 20–30 microspherocytes may be seen per high power field. In severe HS many odd-shaped poikilocytes are also present in addition to spherocytes. Unlike the spherocytes associated with immune hemolytic anemia and thermal injury, most HS spherocytes are fairly uniform in size and density.

The *bone marrow* shows erythroid hyperplasia with normoblasts making up to 25–60% of all nucleated marrow cells. When complicated by folate deficiency megaloblastic features of both myeloid and erythroid precursors are discerned. Bone marrow examination, however, is not needed to diagnose HS.

Osmotic fragility test *(OF):* Red cells serve as osmometers when suspended in varying concentrations of salt solutions, and OF of RBCs is a measure of their spheroidicity. With a decreased surface area relative to volume, sherocytes are unable to bear the introduction of small amounts of free water that occurs when they are placed in progressively more hypotonic solutions. Consequently, spherocytes hemolyze more than the normal discoid RBCs at any salt concentration. Hemolysis is judged by measurning the fraction of total Hb released from RBCs into the extracellular fluid at progressively more dilute salt concentrations. The fresh OF test will detect circulating spherocytes (i.e. these RBCs that have been conditioned by the spleen). This appears as a tail produced by a small population of abnormal cells that undergo hemolysis at salt concentrations that do not affect normal red cells. The most sensitive test to detect HS in the incubated OF test performed after incubating RBCs 18–24 hours understerile conditions at 37°C. This method takes advantage of the fact that all erythrocytes lose membrane under these incubation conditions, however, the process is markedly accelerated in HS red cells. Hemolysis of HS cells may be complete at solute concentrations that causes little or no lysis of normal cells. Also it is not uncommon for some HS individuals to have a normal fresh osmotic fragility (that is, no significant fraction of conditioned RBC), whereas the OF after prolonged incubation at 37°C is usually abnormal. Although OF correlates well with the magnitude of spherocytosis, no correlation is observed between OF and Hb concentration.

As many neonates have few circulating spherocytes and fresh neonatal RBCs normally are relatively resistant to osmotic lysis, therefore when, run with normal neonatal RBC controls, the incubated OF test can be conducted.

Diagnosis

Diagnosis is usually not problematic. A family history of HS or a history of a family member having had a splenectomy or cholecystectomy before the fourth decade of life is usually available. The signs and symptoms of HS are those associated with all chronic hemolytic states (mild pallor, intermittent jaundice, splenomegaly). Classical laboratory features include minimal or no anemia, reticulocytosis, an increased MCHC, normal MCV despite reticulocytosis, spherocytes on the peripheral blood smear, hyperbilirubinemia and an abnormal incubated osmotic fragility test. Other disorders, such as immune hemolytic disease, G6PD deficiency, certain red cell fragmentation syndromes, and thermal and chemical injury to red cells are also associated with spherocytosis. In most of these cases, spherocytes are but one of several types of abnormal RBC present, whereas in HS, there are few abnormal forms other then spherocytes. Additionally, HS spherocytes tend to be more uniform than those noted in other hemolytic anemias. The differentiation between HS and immune hemolytic disease is distinguished by a Coombs' test.

Treatment

The treatment of HS is based upon presplenectomy supportive care, role of splenectomy, and postsplenectomy complications.

Neonates with severe hyperbilirubinemia caused by HS are at a risk of kernicterus, and such infants should be treated with phototherapy and/or exchange tranfusion. Aplastic crises occasionally can cause the hemoglobin level to fall drastically, as the ongoing accelerated destructions of spherocytes is not reciprocated by new RBC production. Under such circumstances red cell tranfusions are often necessary, and may be life-saving. Folic acid is needed to sustain erythropoiesis, and normal dietary folate may be inadequate for children with chronic hemolytic anemia. For this very reason HS patients are instructed to take supplementary folic acid (1 mg/day) in order to prevent the rare megaloblastic crises.

HS is unique among the congenital hemolytic anemias in that splenectomy is permanently curative except in the unusual autosomal recessive variant. Within days of splenectomy, jaundice diminishes and Hb concentration rises. However, red cells survival, although much improved is not absolutely normal. High normal values for reticulocytes and serum bilirubin reflect an ongoing but modest increase in red cell turnover. The numbers of peripheral blood microspherocytes are unchanged or may even increase. The MCV usually falls, but the MCHC does not change significantly. Both leukocytosis and thrombocytosis are expected corollaries of splenectomy. However, postsplenectomy thrombocytosis is not attended by an increased risk of thrombosis, and the use of anticoagulants is not necessary.

Spleen offers critical immunologic protection against certain types of infections; and fatal sepsis caused by *Streptococcus pneumoniae*. The risk of postsplenectomy sepsis is greatest in children less than 5 years of age, although older children and adults also attract an enhanced risk as compared to nonsplenectomised individuals. The estimated rate of mortality from sepsis in splenectomised individuals is 200 times greater than that expected in the general population. The rationale that splenectomy prevents development of gallstone and symptomatic biliary tract disease, as well as obviates the need for major surgery, probably is not as valid today as it was in the past. The availability of laparoscopic cholecystectomy to remove gallstones has had a significant impact on the management of non anemic patients with mild HS who have gallstones. In these patients, if they have no signs or symptoms related to their hemolytic anemia or splenomegaly, it is reasonable to follow them without splenectomy.

If regular ultrasound studies reveal gallstones, it is reasonable to perform a prophylactic laparoscopic cholecystectomy. This procedure will prevent significant biliary tract disease and, in some mild HS patients, obviate the need for splenectomy. Children who are fit cases for splenectomy include those with severe HS requiring red cell transfusions and those with moderate HS who manifest growth failure or other signs and symptoms of anemia. Whenever possible, splenectomy for children with HS should be done after 5 years of age. Partial splenectomy has also been advocated for children less than 5 years of age, this reduces rate of hemolysis and retain splenic immunologic function too. Any child who is to undergo splenectomy must receive pneumococcal vaccine before the spleen is removed. However, as not all pneumococcal subtypes are covered by the vaccine, overwhelming sepsis can still occur in vaccinated children. The most important aspect of this awareness is to have parents alert their physician at the first sign of fever in a splenectomised child. Some clinicians prescribe penicillin prophylactically.

HEREDITARY ELLIPTOCYTOSIS (HE) SYNDROMES (TABLE 8.3)

Hereditary elliptocytosis (HE) syndromes are a group of genetically-determined RBC disorders characterized by elliptical red cells on peripheral smears. By and large, these are clinically silent cases and discovered accidentally on peripheral smear examination. The varied clinical and hematologic manifestations of HE are an expression of numerous molecular defects that give rise to the elliptocyte. Common HE is a dominantly inherited condition characterized by many elliptocytes in the peripheral blood smear. Clinical severity may range from asymptomatic to a moderate hemolytic anemia. Homozygous common HE is a moderate to severe hemolytic anemia, a consequence of homozygosity or double heterozygosity for two common HE mutations. Hereditary pyropoikilocytosis (HPP) is a severe recessively inherited hemolytic anemia, with red cell fragments, microspherocytes, and poikilocytes seen on peripheral smear. Clinically, it is difficult to distinguish HPP from homozygous or doubly heterozygous common HE. Once considered as a separate entity, HPP is biochemically related to common HE, and is now considered to be a variant of this disorder. Spherocytic HE is a rare condition in which both ovalocytes and spherocytes are present on the blood smear. Southeast Asian ovalocytosis (SAO), also known as stomatocytic elliptocytosis, is an HE variant prevalent in the malaria infected belt of southeast Asia and the South Pacific, and is characterized by rigid spoon-shaped cells that have either a longitudinal slit or a transverse ridge.

HE Variants—Prevalence

Not related to any particular race/ethnic group, the prevalence of HE variants is 1 in 1000 to 1 in 5000. HE is more prevalent in malarial belts. HE cells are more resistant to invasion by malarial parasites.

Table 8.3: Clinical classification of HE syndrome

Feature	*Common HE*	*Hemozygous HE*	*Hereditory pyropoikilo-cytosis*	*Spherocytic elliptocytosis*	*Southeast Asian ovalocytosis*
Anemia	None	Moderate-severe	Severe	Mild-moderate	None
Hemolysis	None-mild	Moderate-severe	Severe	Mild-moderate	None
Splenomegaly	None	Present	Present	Present	None
Peripheral blood smear	15–90% elliptocytes	Poikilocytes elliptocytes fragments	Poikilocytosis RBC budding with fragments microspherocytes	Spherocytes elliptocytes	Rounded some having a transverse bar that divides the cell
Osmotic fragility	Normal	Increased	Increased	Increased	Normal-decreased
Inheritance	Dominant	Recessive	Recessive	Dominant	Dominant
Other	Poikilocytosis with severe hemolysis seen transiently in some infants	MCV diminished	MCV diminished		Rigid RBC resistant to penetration by malarial parasite

Pathogenesis of Common HE Disorders

HE disorders are caused by intrinsic membrane protein abnormalities which lead to an alteration in RBC membrane function, change in RBC shape, and in some cases hemolysis.

Membrane Protein Defects

The spectrin extracted from most HE and HPP red cells is quantitatively normal but is structurally abnormal (a major portion of it being dimer, instead of the normal tetramer form). Also, the spectrin isolated from these cells fails to form tetramers in solution. The spectrin dimer–tetramer interconversion is governed by a simple thermodynamic equilibrium which favors spectrin tetramer formation. The impaired self-association of spectrin αβ dimers into tetramers is thought to be responsible for RBC membrane instability in the HE syndromes. Most studies conducted, indicate that HE is associated with dysfunction or deficiencies of spectrin, protein 4.1, and glycophorin.

Spectrin Abnormalities

Over 60% of HE patients show spectrin abnormalities as a result of α-spectrin mutations. In HE, instead of 80 kDa α1 domain peptide, (as obtained from spectrin of normal RBCs) 74 kDa α1 peptide is detected. This peptide is denoted as spα $^{1/74}$, it is an abnormal spectrin and is characterized by abnormalities in domain I of the α chain, a region at the head of α-spectrin involved in dimer-dimer interaction. Spα$^{1/74}$ is the most common of these molecular variants. It is the result of a single amino acid substitution of the α-spectrin chain. Spα$^{1/74}$ is the most severe of the α-spectrin mutations. Spα$^{1/65}$ is a variant that results in a similar, but milder clinical phenotype to that associated with the Spα$^{1/74}$ variant and the mutation is very common. Spα$^{1/46}$ (also identified as Spα$^{1/50}$), occurs primarily in black individuals, but is occasionally reported as a cause of HE in Caucasions.

Although most spectrin mutations are in the vicinity of the αβ-spectrin heterodimer contact, mutations distant from the α1 domain also have been detected. Generally these mutations are asymptomatic in the heterozygous state because they do not affect the spectrin self-association site. A variant Spα$^{v/41}$ has a reduced propensity to form dimers with β-spectrin. When Spα$^{v/41}$ is present by itself, it is clinically silent because normal α D-spectrin is synthesized in large excess of β-spectrin. However, when Spα$^{v/41}$ is present with an α-spectrin mutant that causes HE, incorporation of the later into αβ heterodimers is favored, thereby leading to an increased proportion of the elliptocytogenic spectrin in the red cells. Spα$^{v/41}$ is recognized to have an important role in modifying the clinical expression of HE. This particular polymorphism is common, affecting about 30% of the α-spectrin alleles of Europeans and 20% of Japanese and African Blacks.

Individuals with a single copy of the variant α-spectrin genes generally have asymptomatic elliptocytosis, however, those with two copies have severe hemolytic anemia. The clinical phenotype produced by α-spectrin mutants is determined by two factors; the relative amount of spectrin present as dimer and the total spectrin content of the membrane. In HE cells, the percentage of spectrin content is normal; in HPP red cells, the percentage of spectrin dimers also is high, but the spectrin content is decreased.

Protein 4.1 Defects

Protein 4.1 abnormalities associated with HE are much less common than spectrin mutations. One site on protein 4.1 binds to the distal end of the spectrin αβ heterodimer, thereby increasing the binding of spectrin to actin. A second domain is where 4.1 interacts with glycophorin C, thereby enhancing attachment of spectrin to the membrane. Protein

4.1 deficiency has been reported from France and northern Africa. A partial deficiency of protein 4.1 is associated with mild dominantly inherited HE. Heterozygotes have approximalety 50% of the normal amount of protein 4.1 and have a mild hemolytic anemia. Homozygotes have no detectable protein 4.1 and a severe transfusion-dependent hemolytic anemia with prominent elliptocytosis and spherocytosis.

Glycophorin C Deficiency

Mutations associated with glycophorin C deficiency occasionally are associated with HE. These cases usually include partial deficiency of protein 4.1. Glycophorin C is decreazed in patients with protein 4.1 deficiency, presumably because the two proteins stabilise each other on the membrane. In contrast to other form of HE, heterozygous carriers of glycophorin C deficiency are asymptomatic with normal RBC morphology-homozygous deficient individuals have mild elliptocytosis but no anemia.

Elliptocyte Formation due to Membrane Abnormalities

Due to spectrin mutations and/or protein 4.1 deficiency the horizontal integrity of the membrane is disturbed; consequently cellular deformability and membrane fragility are deranged. The basis for elliptocytosis and mechanical instability of glycophorin C deficient red cells is not known. It appears to be related to a concomitant partial deficiency of protein 4.1. The fault is corrected (wherever protein 4.1 is deficient) by introduction of protein 4.1 into the cells.

The exact mechanism by which these protein defects result in elliptocyte formation is not clear. The HE red cells acquire their shape after release from the bone marrow and as they age *in vivo.* The normal red cells assume elliptical shape while passing through the microcirculation and *in vitro* when they are exposed to shear stress; and that with passage through microcirculation or with removal of shear stress, they resume a normal biconcave shape. However, if mechanical distortion is maintained for long periods, even normal red cells remain elliptocytic, thus suggesting that the membrane skeleton is altered as a consequence of sustained shape changes. In the case of HE, it is possible that weakened spectrin hetrodimer contacts may lead to skeletal reorganization after less extensive deformation or shear stress. With repeated distortions experienced by passage though small capillaries, cells with unstable membrane skeletons thus may gradually elongate to form irreversible elliptocytes. Red cells with severe skeletal defects may not be able to withstand the shear stresses experienced in normal circulation, and thus may udergo fragmentation. This process may explain the poikilocytosis seen in homozygous HE, HPP and in mild HE associated with neonatal poikilocytosis. In all of the hemolytic HE syndromes, the more severely distorted cells are retained in splenic cords. Consequently, splenectomy profoundly imporves the anemia assoicated with these clinically symptomatic variants.

Clinical and Laboratory Findings of HE Syndromes

Though all HE variants are, marked by the presence of at least 15% elliptical red cells, they are clinically and hematologically quite heterogenous.

Common HE (Fig. 8.3)

Usually discovered accidentally on peripheral smear examination. Individuals are usually not anemic, with normal red cell survival and do not have splenomegaly. In contrast to the clinical picture, the peripheral smear is striking, containing 15–100% elliptocytes. Other common HE cases involve mild compensated hemolysis without anemia.

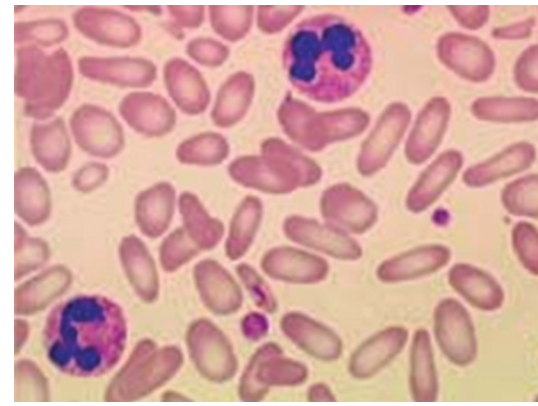


Fig. 8.3: Hereditary elliptocytosis, peripheral smear

However, 5–20% of cases have incompletely compensated hemolysis and patients have a moderate to severe hemolytic anemia (Hb = 9–12 g/dL and reticulocyte count = 20–25%). The peripheral blood in these cases reveals many poikilocytes, as well as numerous elliptocytes. There may be splenomegaly with gallstones. Abrupt episodes of acute anemia may occur. The genetic variations which lead to variable clinical pictures have been explained earlier in this chapter. Hereditary elliptocytosis with infantile poikilocytosis is a clinical manifestation of HE that occurs almost exclusively in black families. Affected young infants have moderately severe hemolytic anemia and hyperbilirubinemia in the neonatal period, the latter often necessitating exchange transfusion. The peripheral smear shows marked red cell fragmentation and poikilocytosis in addition to elliptocytosis. These morphologic changes are indistinguishable from those noted in patients with HPP. In contrast to HPP, however, hemolysis gradually lessens, and the clinical and hematologic features convert to those of mild HE by 6–12 months of age. The exact basis for this curious phenomenon is not known.

Homozygous (Doubly Heterozygous) HE

Homozygous and doubly heterozygous HE are characterized by chronic hemolytic anemia of variable severity. Morphologic characteristics include marked poikilocytosis, microelliptocytosis, and red cell fragmentation. In many cases both parents have nonhemolytic HE, or because many individuals with a single α-spectrin mutation are asymptomatic, parental anemia or RBC abnormalities may not be present. These disorders and HPP have a very similar clinical presentation. The homozygous state for the $Sp\alpha^{1/74}$ variant and the $Sp\alpha^{1/65}$ variant have been described. These individuals homozygous for $Sp\alpha^{1/65}$ have moderate hemolysis, whereas those patients homozygous for $Sp\alpha^{1/74}$ mutation have much more severe hemolytic anemia, often requiring splenectomy. The latter homozygous group of HE subjects may have numerous small microspherocytes in their peripheral blood and their clinical presentation is indistinguishable from HPP. Several cases presumed to result from homozygosity now are known to be a consequence of double heterozygosity for different spectrin mutations.

HEREDITARY PYROPOIKILOCYTOSIS (HPP) (FIG. 8.4)

A severe congenital hemolytic anemia, which clinically is very similar to homozygous HE, is characterized by extreme micropoikilocytosis and an autosomal mode of inheritance. HPP is the result of a double heterozygous state for an α-spectrin mutant and a defect involving spectrin synthesis. HPP is now considered as a subtype of common HE. The one fundamental difference between these two entities is that red cells of the HPP subjects also are partially deficient in spectrin as detected by a decreased spectrin to band 3 ratio. To an extent this may reflect that some elliptocytogenic α-spectrin mutants are unstable, and this might lead to spectrin deficiency. Irrespective of the mechanism, decreased red cell spectrin is a feature of HPP and probably explains the abundance of microspherocytes noted in association with this disorder. In infancy or early childhood, affected individuals manifest

moderately severe hemolytic anemia (Hb = 5–9 g/dL) (reticulocytosis 13–35%). Splenectomy is followed by an increase in hemoglobic concentration (9–11 g/dL) and a decrease in reticulocytosis (3–7%). Usually blacks are involved. Growth retardation related to anemia, frontal bossing, and premature gallbladder disease are usual complication. Most profound are changes in red cell morphology, which include extreme poikilocytosis, microspherocytosis, microelliptocytosis, membrane budding and red cell fragmentation. The MCV may be extremely low, 30–50 fL, MCHC is normal and osmotic fragility is increased.

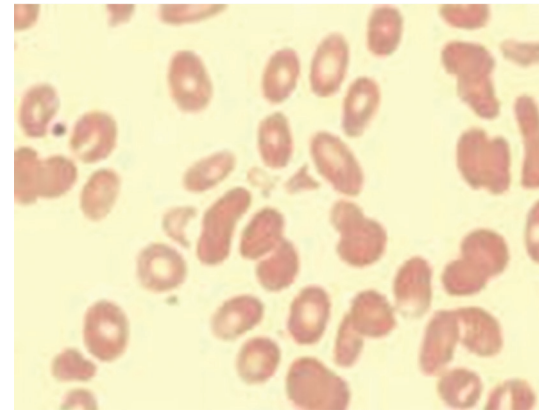


Fig. 8.4: Hereditary pyropoikilocytosis

Spherocytic Elliptocytosis (SE)

It is characterized by two population of cells, red cells that are more rounded than typical hereditary elliptocytes and a variable number of microspherocytes. Here no poikilocytes or red cell fragments are seen. SE comprises about 15–25% cases of HE-in Caucasians. Affected individuals have an incompletely compensated hemolytic process with mild anemia and tendency to aplastic crises. The relative numbers of spherocytes and elliptocytes are variable. As in HS, the increased. Splenectomy is causative. Homozygosity for SE is characterized by severe hemolytic anemia, striking microspherocytosis and poikilocytosis, and an excellent response to splenectomy.

Southeast Asian Ovalocytosis (SAO)

Also known as stomatocytic elliptocytosis, is characterized morphologically by round elliptocytes (ovalocytes), many of which have one or two transverse ridges. These red cells are found in people from Malaysia, New Guinea, Indonesia, and the Philippines. Their presence is not associated with any significant anemia or hemolysis. The RBCs in SAO manifest increased red cell rigidity, increased thermal stability, and decreased osmotic fragility. Also these red cells are resistant *in vitro* to invasion by several strains of malaria. In populations where SAO is prevalent, the frequency of the morphologic abnormality is increased in older individuals, thus suggesting a protective effect this RBC anomaly. The molecular defect in SAO results from an abnormal band 3 protein. All carriers of the SAO phenotype are heterozygotes, with one normal band 3 allele, and one mutant band 3. Tight binding of band 3 protein to ankyrin is present in SAO red cells; this restricts lateral mobility of this protein. Resistance of SAO red cells to malaria is most likely related to the band 3 protein because this protein is a receptor for the parasite. The reduced lateral mobility of band 3 protein in SAO red cells may limit invagination and penetration by the parasite.

Laboratory Findings

One should assess:

1. Hemoglobin/PCV for anemia.
2. Compensation of anemia (reticulocyte count).
3. Serum LDH and bilirubin (accelerated RBC destruction).

4. MCV—important because hemolytic variants with RBC fragmentation often manifest severe microcytosis because of RBC fragmentation.
5. Peripheral smear examination—very essential for diagnosis of HE and the classification of the disorder into major subtypes. At least 15% and often as many as 50–90% of red cells are oval, whereas smears from normal subjects contain fewer than 15% elliptocytes. Where elliptocytosis is the only morphologic abnormality, hemolysis is usually minimal or absent with the exception of spherocytic elliptocytosis, in which the presence of "fat" ovalocytes is associated with accelerated red cell destruction. In other patients with hemolytic forms of HE, poikilocytosis almost always is seen on the blood film, and in homozygous HE and HPP, many red cells circulate as red cell fragments, producing a marked decrease in MCV. The finding of red cell fragments together with a striking microspherocytosis, and often only occasional elliptocytes is characteristic of HPP.
6. Osmotic fragility is normal in nonhemolytic HE, but is increased in spherocytic elliptocytosis, HPP, and in those hemolytic HE variants with poikilocytosis on their peripheral blood film. OF is decreased (that is, resistant to hemolysis) in SAO red cells.

SAO is characterized by the finding of over 30% oval red cells on the peripheral blood film, some containing a central slit or a transverse ridge, together with an absence of clinical and laboratory evidence of hemolysis. A screening test for SAO is the failure of ovalocytes to undergo membrane changes (form spicules) following metabolic depletion or when suspended in hypertonic salt solutions. The mechanism of this resistance to change in shape presumably is because of the extreme rigidity of the red cell membrane. As mentioned earlier, the osmotic fragility is decreased.

Once the diagnosis of HE syndrome is established, additional laboratory studies (electrophoretic) can further classify the cellular and molecular defects.

Treatment

On account of benign nature of autosomal dominant HE, therapeutic intervention is not indicated usually. SAO affected individuals also do not need any therapy. However, patients with variants that are associated with chronic hemolytic anemia (homozygous HE, HPP, spherocytic HE) may benefit from splenectomy. In HE cases associated with hemolysis, red cells are congested in the splenic cords and splenectomy is followed by improvement or normalisation of the hemoglobin concentration and decrease in the reticulocyte count, despite persistence of elliptocytosis.

STOMATOCYTIC DISORDERS

Stomatocytes are red blood cells with a central slit as stoma instead of a circular pallor when observed on dried smears. In wet preparations, they are uniconcave instead of being biconcave, giving them a bowl-like appearance. *In vitro*, stomatocytes are produced by drugs that intercalate into the inner half of the lipid bilayer, thereby expanding the inner lipid surface area relative to that of the outer half of the bilayer. Echinocytes (RBCs with numerous fine spicules) are thought to be the result of preferential expansion of the outer lipid bilayer relative to the inner layer. A few stomatocytes may be found in blood smears prepared from normal individuals, as well as patients with acute alcoholism and hepatobiliary disease. Also aquired stomatocytosis occurs in individuals receiving vinca alkaloids (velban, vincristine). Large number of stomatocytes are associated with rare hereditary disorders of red cells cation permeability leading to increased red cell water content (hereditary stomatocytosis) or cellular dehydration (hereditary xerocytosis).

Stomatocytosis also is associated with absence of Rh blood group antigens on the red cell membrane (Rh null phenotype). Each of these stomatocytic disorders is associated with mild to moderate hemolysis.

To understand the pathophysiology of hereditary stomatocytosis and hereditary xerocytosis, a clear knowledge of normal red cell cation and water transport mechanism is necessary. The RBC membrane, which is freely permeable to water, controls its volume primarily through regulation of the monovalent cation content. Small passive cation leaks (sodium in, potassium out) are normally balanced by the active outward transport of sodium (3 mEq per liter of red cells per hour). These cation pumps are linked, require ATP, and are dependent on the membrane enzyme Na-K ATPase. If the membrane permeability leak to monovalent cations increases, cation pumps have limited compensatory ability and, if this capacity is exceeded, red cell volume changes in parallel with the total cation change. Red cells swell when the inward sodium leak exceeds the potassium leak out; and red cells shrink when the potassium leak out exceeds the inward sodium leak. These membrane permeability abnormalities are recognized by observing altered RBC hydration (decreased or increased MCHC) and altered red cell sodium, potassium, and total monovalent cation content.

Hereditary Stomatocytosis (Fig. 8.5)

Hereditary stomatocytosis (or hereditary hydrocytosis refers to a heterogeneous group of autosomal dominant hemolytic anemias caused by altered sodium permeability of the red cell membrane. The important pathophysiologic abnormality is the result of a marked increase in sodium permeability (15–40 times normal), and this leads to increase in RBC sodium, a relatively lesser decrease in intracellular potassium, an increase in total monovalent cation content, and thereby an increase in cell water. Despite a marked compensatory increase of active sodium and potassium transport, increased pump activity is unable to compensate for the markedly increased inward sodium leak. Treatment of these cells with dimethyl adipimidate (a bifunctional imidoester which cross-links proteins) normalizes membrane permeability, corrects cell cation and water content, improves membrane permeability, and corrects abnormal RBC morphology. Moreover, these treated RBCs have an improved survival *in vivo.* These cases are thought to be occurring due to absence of "stomatin" (or band 7.2). However, the function of this protein currently is not understood, but its structural gene has been isolated and cloned.

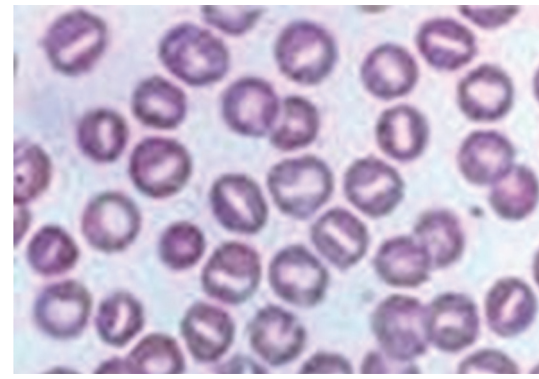


Fig. 8.5: Hereditary stomatocytosis, peripheral smear

The severity of this disease is variable from insignificant to one requiring transfusion. Characteristically, anemia is mild. Reticulocytosis is moderate (10–20%) in most patients but wide variations are found. About 10–50% of circulating red cells are stomatocytic. Osmotic fragility and MCV are increased, while MCHC is normal or low (as a result of increased cell water content). Measurement of red cell sodium (increased) and potassium (decreased) will identify these anomalies. In most patients, hemolytic anemia is improved following splenectomy.

Hereditary Xerocytosis (Fig. 8.6)

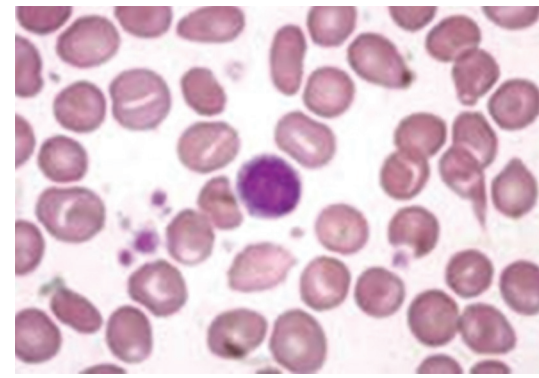


Fig. 8.6: Hereditary xerocytosis

Hereditary xerocytosis (or hereditary desiccocytosis) is a rare autosomal dominant hemolytic anemia characterized by red cell dehydration and decreased osmotic fragility. The mechanism of cellular dehydration involves net potassium loss from cells that is not accompanied by a proportional gain of sodium. Measurement of RBC cation reveals a slightly raised cell sodium content, a greater decrease in potassium concentration, and thus a decrease in the net intracellular cation content and cell dehydration. Various reasons thought to be operating are reduced red cell 2, 3 DPG, an increase in RBC phosphatidyl choline and elevated membrane lecithin. However, the exact reason for increased leakiness of the membrane to potassium is not known. There is no known membrane protein defect. The RBCs in hereditary xerocytosis are sensitive to shear stress, and readily undergo membrane fragmentation. These patients typically have moderate to severe hemolysis. The MCHC is increased, indicating cellular dehydration, whereas MCV is slightly increased, on account of reticulocytosis. Peripheral blood smear reveals stomatocytes, target cells, spiculated cells, and some cells in which hemoglobin is concentrated in discrete areas of the cell pariphery. The osmotic fragility is characteristically decreased. Splenectomy has no or little effect on the hemolytic anemia.

Intermediate Stomatocytic Syndromes

Some cases show featuers of both hereditary stomatocytosis and xerocytosis. Affected individuals have stomatocytes and target cells on the peripheral blood smear. The osmotic fragility is either normal or slightly increased. The sodium and potassium permeability is somewhat increased but the intracellular cation content and the RBC volume usually are normal or slightly reduced. In the variant known as cryohydrocytosis, RBCs *in vitro* at 5°C gain sodium and exhibit greater autohemolysis than at 37°C. In another type, potassium loss is accelerated in the cold, and blood samples left at room temperature for a few hours may manifest pseudohyperkalemia because of potassium efflux from the RBC into the plasma. In contrast to cryohydrocytosis, these RBCs in the cold manifest greater potassium loss than sodium gain, and thereby lose water (cryoxerocytes). Despite the mildly dehydrated red cells, no anemia or hemolysis is associated with this defect.

Rh Null Disease

Rh null hemolytic disease occurs in those rare individuals who have no Rh antigen expression, and this is different from the Rh negative phenotype (cde) where Rh proteins are present. The Rh proteins (D, Cc, Ee) are linked to the RBC membrane skeleton, but their function is unknown.

Rh null disease is characterized by mild-tomoderate normocytic, normochromic hemolytic anemia. The hemoglobin concentration is decreased (8–13 g/dL) and reticulocytosis (6–20%) occurs. Numerous stomatocytes and some spherocytes are seen on peripheral blood smear. The red cell monovalent cation and water content is decreased, and these cells are dehydrated. However, the osmotic fragility of incubated cells is increased. Thus reflecting a marked reduction of membrane surface area. The half-survival time

of ^{51}Cr-labeled RBCs is 10–12 days, but the reason for shortened red cell survival is not understood. Hemolytic anemia improves following splenectomy.

ACANTHOCYTIC DISORDERS (FIG. 8.7)

These red cells are dense and contracted with several irregularly spaced "thorny" projections on the surface. Acanthocytes differ from echinocytes in that there are fewer projections, and the width and length of these projections vary considerably. No central pallor is visible. By contrast, echinocytic spicules are similar in dimension and evenly distributed. Acquired acanthocytosis is found in malnourished states such as anorexia nervosa, in hypothyroidism, after splenectomy, and in severe hepatocellular disease. Acanthocytosis is also seen in hereditary disorders such as abetalipoproteinemia, and in patients with the MacLeod phenotype.

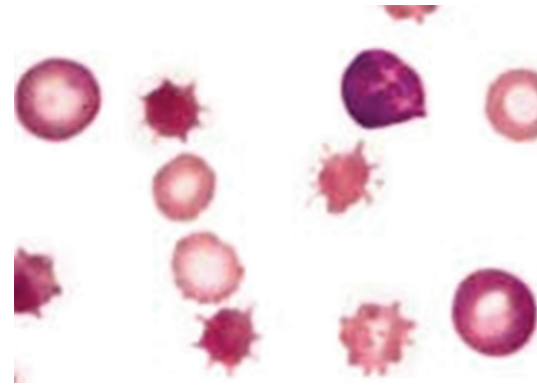


Fig. 8.7: Acanthocytes

Acanthocytosis in liver disease (hepatocellular injury) is attributable to a marked increase in the cholesterol content and the cholesterol to phospholipid ratio of red cell membranes. This red cell lipid profile is in contrast to the equal increase in both cholesterol and phospholipid seen in obstructive liver disease. In cirrhotic liver disease, abnormal lipoproteins produced by the liver are loaded with cholesterol; and this excess cholesterol is readily transfered to RBCs, resulting in the formation of flat scalloped cells. These cells, however, are further conditioned by the spleen, resulting in membrane fragmentation, and evolution of acanthocytes which look like "spurs". These spur RBCs are cholesterol-loaded, with a decreased surface area to volume ratio, and their cellular deformability is decreased. Once formed in spleen, spur cells over time are destroyed by the spleen, thereby accounting for the hemolytic anemia associated with severe liver disease. Affected patients usually have moderate to severe hemolysis, hyperbilirubinemia, splenomegaly, and clinical and laboratory evidence of liver dysfunction. The peripheral blood contains numerous acanthocytes, very similar in appearance to those seen in abetalipoproteinemia. This spur cell anemia can occur in any condition associated with severe liver injury.

Abetalipoproteinemia

Abetalipoproteinemia (or Bassen-Kornzweig syndrome) is a rare, genetic disorder characte-rized by acanthocytosis, fat malabsorption, hypolipidemia, retinitis pigmentosa and progressive ataxia. The primary biochemical lesion results from defective synthesis of apoprotein B, and, consequently, an absence of several lipoprotein fractions; low density lipoproteins (LDL), very low density lipoprotein (VLDL), and chylomicrons. Also, the metabolic activities of these lipoprotein (mobilization of trighycerides from the liver, transport of phospholipid and cholesterol between phospholipid, and triglyceride levels are very low; and serum appears transparent. Plasma lipid disturbance, as related to acanthocyte formation is not understood. Total red cell lipid and phospholipid content are normal, but there is a net increase in sphingomyelin concentration, thereby reversing the usual phosphatidylcholine to sphingomyelin ratio. This phospholipid distribution is similar to that seen in the plasma where an identical increase in sphingomyelin exists relative to phosphatidyl choline. RBC precursors and young erythrocytes are of normal

shape in this disorder. Acanthocyte formation increases with RBC age in the circulation. Moreover, normal red cells become acanthocytic when infused into individuals with abetalipoproteinemia. Unlike acanthocytes produced in liver disease, those from subjects with abetalipoproteinemia do not revert to a discoid shape when incubated in normal serum. However, this acanthocytosis is reversed *in vitro* after lipids are extracted with detergents. The reason for acanthocyte formation is thought to reflect an increase in the surface area of the outer lipid bilayer relative to the inner bilayer, presumably because of increased sphingomyelin in the outer bilayer leaflet. Acanthocytes do resume a discocyte morphology after incubation with chlorpromazine, an agent known to expand the inner lipid bilayer, thereby normalizing the bilayer asymmetry. The affected infants are normal at birth but the clinical features, *viz.*, steatorrhea, abdominal distention, and growth failure develop in the first month of life. Retinitis pigmentosa and progressive ataxia are first noticed in children between 5 and 10 years of age. Neurologic disability is progressive, with death usually occurring during the second or third decade of life. In a few cases, cardiac arrhythmias and heart failure precede death. As several cases of this disorder have been seen among consaguinous parents, an autosomal recessive mode of inheritance is assumed.

The anemia in abetalipoproteinemia is mild to nonexistent—in contrast to the spur cell anemia of liver disease. Nutritional deficiencies, if associated may markedly aggravate hemolysis in abetalipoproteinemia too. The labelled RBCs here show just a slightly shortened lifespan. Reticulocyte counts are normal or slightly raised. The most striking feature is the presence of large numbers of acanthocytes (50–70%) on the blood smear. Osmotic fragility is normal or slightly decreased.

The diagnosis established by observing acanthocytes on peripheral smear, clinical features and cholesterol, phospholipid and triglyceride levels (all are very low). The diagnosis is confirmed by demonstrating the absence of betalepoproteins in plasma.

MacLeod Phenotype

MacLeod phenotype is characterized by a decreased expression of the Kell antigen on the red cell surface, and decreased reactivity to Kell antiserum. It is the result of an absence of the Kx antigen, a protein precursor necessary for the Kell antigen expression. The gene controlling Kx synthesis is not part of the Kell autosomal gene complex, but rather is located on the X-chromosome. This rare anomaly of the Kell system is known as the MacLeod phenotype, named after the man whose red cells showed weak reactivity to Kell antisera. It is associated with a disorder characterized by acanthocytosis, polychromasia, a mild, well-compensated hemolytic anemia, and susceptibility to immunisation by Kell antigens. Acanthocytosis is to the tune of 25–85% of the RBCs. The reason, here too, is thought to be asymmetry of the lipid because agents that expand the inner lipid layer correct the acanthocytosis. Studies of membrane lipid and protein compositions membrane fluidity, intercellular enzyme at ATP levels are normal.

The Kx antigen is present in trace amounts on normal red cells and is strongly active on normal neutrophils and monocytes. The MacLeod syndrome occurs in association with chronic granulomatous disease of childhood, retinitis pigmentosa and Duchenne muscular dystrophy. This association strongly reflects the close proximity of the genetic loci of these conditions in the p21 region of the X chromosome, and the deletion of Xp21 leads to the diseases mentioned previously. The patients of MacLeod phenotype (boys) have an increased number of reticulocytes and shortened red cell survival; some have required transfusions. Their carrier mothers have a dual population of Kx positive and Kx negative erythrocytes and variable number of acanthocytes.

Echinocytic Disorders (Fig. 8.8A)

RBCs with numerous fine uniform spicules equally throughout the cell surface are called echinocytes. These cells differ morphologically from acanthocytes, which have fewer projections, and the spicules vary in size. It is thought that echinocytes are the result of preferential expansion of the outer lipid bilayer relative the inner layer. As mentioned earlier, stomatocytes, in contrast, are produced by agents that expand the inner lipid surface area relative to the outer half of the bilayer. The presence of echinocytes on the peripheral blood film often is an artifact, caused by interactions of red cells with glass. However, echinocytes also are seen in association with hemolytic anemias, in patients with hypophosphatemia, in patients with pyruvate kinase deficiency, in uremic patients, and in some long distance runners. The exact mechanism of echinocytosis in these diverse conditon is not clear. However, *in vitro* it is known that a variety of factors, such as exposure of red cells to certain drugs, loading by calcium, or ATP depletion all can cause echinocyte formation.

Target Cell Disorders (Fig. 8.8B)

These are discoid RBCs with a centralized hemoglobinized area in the clear center, thereby resembling a shooting target. Target cells are the morphologic expression of an increase in the ratio of cell surface area to cell volume. This ratio is influenced by increases in the surface area, as well as decreases in cell volume. Causes of target cell formation may be both acquired and hereditary. In microcytic cells of iron deficiency, thalassemias, and certain hemoglobinopathies, a decreased cell volume caused by less hemoglobinization results in relative excess of cell surface area. Therefore, the surface area to volume ratio is increased, and hence the expression of target cells. In obstructive liver disease and disorder of intrahepatic cholestasis, target cell formation is the result of an absolute increase of the cell-surface area because of net membrane accumulation of both phospholipids and cholesterol. The accumulation is caused by abnormal low density lipoprotein which occur in obstructive jaundice; these LDL proteins are laden with cholesterol and lecithin which is readily transferable to red cell membrane, thereby leading to an expansion of the cell membrane surface. Target cells have a decreased osmotic fragility as the excess of membrane surface area leads to increase of the critical hemolytic volume. These target cells have a normal survival in circulation.

Familial Lecithin-cholesterol Acyl-transferase (LCAT) Deficiency (Fig. 8.8C)

Lecithin cholesterol acyltransferase (LCAT) catalyses the transfer of fatty acids from phosphatidylcholine to cholesterol. This enzyme is in the circulation complexed with high density lipoproteins (HDL). Familial LCAT deficiency is a rare disorder characterized by

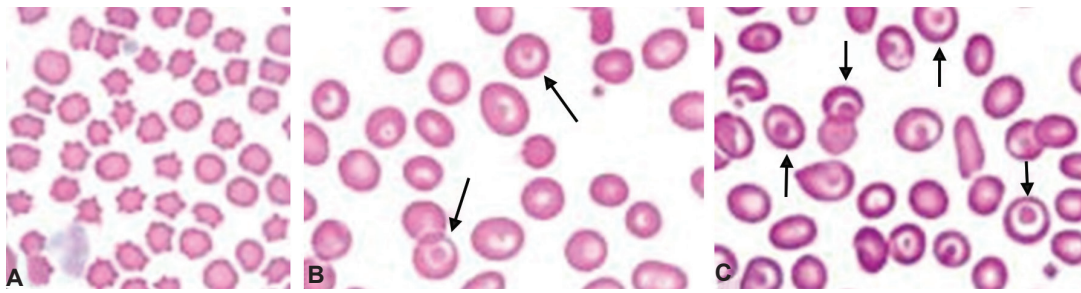


Fig. 8.8: (A) Echinocytes in peripheral smear; (B) Target cells as seen in peripheral smear; (C) LCAT deficiency, peripheral smear picture

mild anemia, corneal opacities and renal disease. Results of family studies support an autosomal recessive mode of transmissin. LCAT deficiency is responsible for a marked decrease in plasma levels of unesterified cholesterol and an increase in the amount of free cholesterol. The red cell membrane may contain twice normal amounts of unesterified cholesterol; but phosphatidylcholine also is increased whereas sphingomyelin and phosphatidylethanolamine are reduced. These RBC changes are reversible when the target cells are incubated with normal plasma. The anemia is mild, and target cells are prominent on the peripheral blood smear. Both hemolysis and decreased erythropoiesis have been implicated in the pathogenesis of the anemia, the later may relate to coexistent renal disease. Foam cells and "see blue histiocytes" have been noted in both the bone marrow and the spleen of all patients studied. The material accumulated within histiocytes is possibly unesterified cholesterol and phosphatidylcholine.

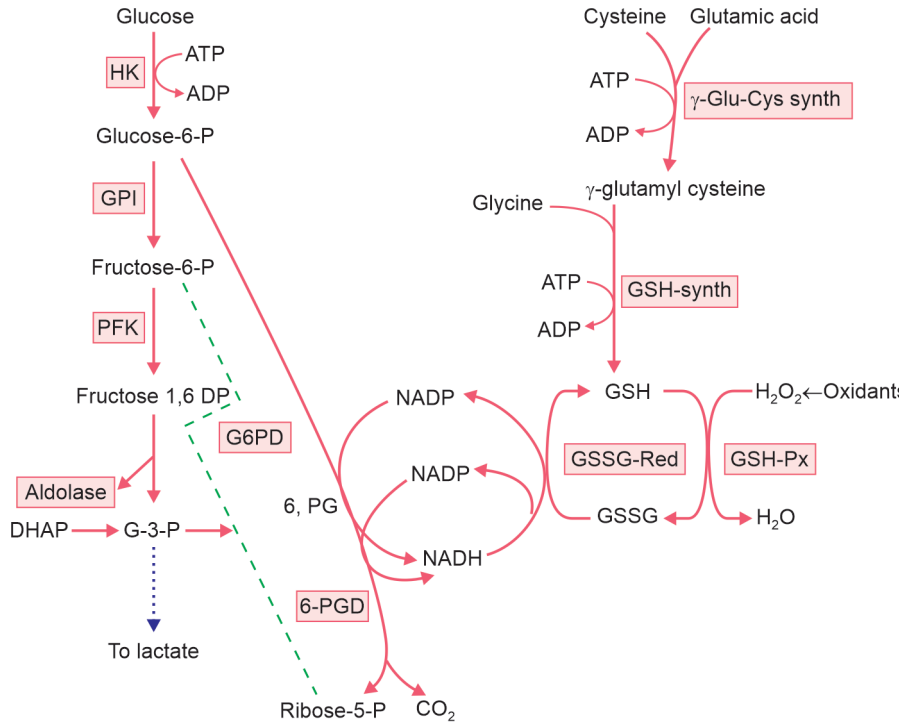
GLUCOSE-6 PHOSPHATE DEHYDROGENASE DEFICIENCY AND RELATED DISORDERS OF HEXOSE MONOPHOSPHATE SHUNT AND GLUTATHIONE METABOLISM

The hexose monophosphate (HMP) shunt pathway metabolises 5–10% of glucose used by RBCs, and this is critical for protecting red cells against oxidant injury. The HMP pathway is the only RBC source of reduced nicotinamide adenine dinucleotide phosphate (NADPH), a cofactor important in glutathione metabolism. Red blood cells contain high concentration of reduced glutathione(GSH), a sulfhydryl containing tripeptide (glutamyl cysteinglycine), which functions as an intracellular reducing agent that protects cells against oxidant injury. Oxidants such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), are produced by exogenous factors (such as drugs and infections) and are also formed within red cells as a consequence of reactions of hemoglobin with oxygen. However, when these oxidants accumulate within red cells, hemoglobin and other proteins are oxidized, leading to loss of function and RBC death. Under normal circumstances this does not occur because GSH, in conjuction with the enzyme glutathione peroxidase (GSH-Px) rapidly inactivates these compounds. During the oxidant detoxification process, however, GSH itself is converted to oxidized glutathione (GSSG), and GSH levels fall. In order to sustain protection against persistent oxidant injury, GSH levels must be maintained, and this is accomplished by glutathione reductase (GSSG-Rx) which catalyses the reduction of GSSG to GSH. This reaction requires the NAD-PH generated by glucose 6-phosphate dehydrogenase (G6PD), the first enzymatic reaction of the HMP shunt. Therefore, the tight coupling of the HMP shunt and glutathione metabolism is responsible for protecting intracellular proteins from oxidative assault. Almost all hemolytic episodes related to altered HMP shunt and glutathione metabolism are caused by G6PD deficiency, and this enzyme deficiency is known to affect millions of people throughout the world. Rare cases of hemolysis associated with decreased GSSG-Rx activity, GSH-Px deficiency and deficiencies of GSH synthetic enzymes also have been described (Flowchart 8.3).

GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY

G6PD catalyses the initial step in the HMP shunt, and is now known to be critical in protecting RBC from oxidant injury. In most patients with G6PD deficiency, there is no anemia in the steady state, reticulocyte counts are normal, but RBC survival may be slightly diminished, however, episodic exacerbations of hemolysis accompanied by anemia occur in association with the administration of certain drugs and with some infections. In a few cases, G6PD deficiency is associated with a chronic hemolytic process. Till date, over 400 G6PD variants have been recognized.

Flowchart 8.3: HMP shunt, enzymes of glutathione (GSH) metabolism, protection from oxidant assault and relationship to glycolytic metabolism



Hk = Hexokinase, GPI= Glucose phosphate isomerase, PFK = Phosphofructokinase, G-6-PD = glucose-6-P dehydrogenase, 6,PGD=6, phosphogluconate dehydrogenase, GSSG-Red = glutathione reductase, GSH-Px = glutathione peroxidase, GSH-synth = glutathione synthetase, ϑ-Glu-Cys-Synth = ϑ glutamyl-cysteine-synthetase G-3-P = glyceraldehyde-3-phosphate, DHAP = dihydroxy acetone phosphate, Ribose-5-P = Ribose-5-Phosphate, 6,PG=6, phosphogluconate GSH = reduced glutathione, GSSG = oxidized glutathione.

Genetics

The G6PD gene is located on the X chromosome (band X q 28). The fact that normal males and females have the same enzymes activity in their red cells is explained by the Lyon hypothesis. This hypothesis maintains that one of the two X chromosomes in each cell of the female embryo is inactivated and remains inactive throughout subsequent cell division for the duration of life. Enzyme deficiency is expressed in males carrying a variant gene, whereas heterozygous females usually are clinically normal. However, depending on the degree of lyonization and the degree to which the abnormal G6PD variant is expressed, the mean RBC enzyme activity in female may be normal, moderately reduced or grossly deficient. A female with 50% normal G6PD activity has 50% normal red cells and 50% G6PD deficient red cells. However, the G6PD deficient cells in females are as valuable to hemolysis as are enzyme deficient red blood cells in males.

Prevalence and Geographic Distribution

Over 20 crore people are affected worldwide (mainly in the tropical and subtropical zones of the eastern hemisphare). About 2.6% of Indian males are thought to be affected. Because of the high incidence among populations in which malaria was endemic, G6PD deficiency is thought to confer a selective advantage against infection by falciparum malaria. The

parasitic growth is inhibited in the G6PD-deficient red cells. Why? One possibility is that the oxidant stress that causes GSH instability and destroys the host RBC also kills the parasite. An alternative explanation is provided by the observation that infected, G6PD-deficient red cells are unable to generate the ribose derivatives needed by the parasite for nucleic acid synthesis. Thus, inhibition of parasite growth, rather than oxidative destruction of the parasite or host cells, may be an important mechanism for the balanced polymorphism of the G6PD gene.

G6PD Enzyme and its Variants

The monomeric form of G6PD contains 515 amino acids and has a molecular weight of over 59,000 Da. The active form of G6PD *in vivo* is a dimer that contains tightly bound NADP. The normal or wild type enzyme is G6PD B, although hundreds of variant enzymes have been identified. Currently about 442 distinct forms of G6PD are recognized (overlapping of distinct forms is a possibility).

The WHO has further classified the different G6PD variants on the magnitude of the enzyme deficiency and also the severity of hemolysis. Class I variants have very severe enzyme deficiency (<10% of normal) and have chronic hemolytic anemia. Class II variants also have severe enzyme deficiency, but there is usually only intermittent hemolysis. Class III variants have moderate enzyme deficiency (10–60% of normal), with intermittent hemolysis usually associated with infection or drugs. Class IV variants have no enzyme deficiency or hemolysis. Class V variants are those in which enzyme activity is increased. Variants in the last two groups although of much interest to biologists, geneticists and anthropologists, are of no clinical significance.

The normal wild type enzyme, G6PDB, is found in most Caucasians, Asians and a majority of blacks. It has normal catalytic activity and is not associated with hemolysis (class IV). A commonly encountered variant is G6PD A^+, which is found in 20–30% of blacks from Africa. It has normal catalytic properties and does not cause hemolysis (class IV). It differs from G6PD B in that it has a much faster electrophoretic mobility. Another common variant is, G6PDA^- is the enzyme responsible for primaquine sensitivity in blacks, and it is the most common variant associated with mild-to-moderate hemolysis (Class III). This G6PD variant is found in 10–15% of African Americans and with similar frequencies in western and central Africa. It has an electrophoretic mobility identical to that of G6PD A^+. However, this is an unstable enzyme and its catalytic activity although nearly normal in bone marrow cells and reticulocytes decreases markedly in older RBCs. Hence, this variant is designated G6PD A^- (the + and –denote enzyme activity). G6PD Mediterranean is the most common abnormal variant found in Caucasians, particularly those whose origins are in the Mediterranean area. Its electrophoretic mobility is identical to that of G6PD B, but it is synthesized at a reduced rate, its catalytic activity is markedly reduced and hemolysis can be severe (Class II). G6PD Canton is a variant enzyme seen in Asians. Its biochemical properties are very similar to those of G6PD Mediterranean.

Because leukocyte and platelet G6PD is regulated by the same gene as not of red cells, documentation of decreased activity the WBCs and platelets of deficient subjects is not surprising. Because of normally short survival of WBCs and platelets, however, subjects with unstable variants rarely manifest functional impairment. Phagocytic and bactericidal activity of granulocytes from deficient subjects are characteristically normal.

Pathophysiology

As red cells age, the activity of G6PD declines exponentially. The normal enzyme (G6PD B) has an *in vivo* half-life of 62 days. Despite this loss of enzyme activity, normal old RBCs

contain sufficient G6PD activity to generate NADPH and thereby sustain GSH levels in the face of oxidant stress. In contrast, G6PD variants with hemolysis have much shorter half lives. The activity of G6PDA$^-$ in reticulocytes is normal, but it declines rapidly thereafter with a half-life of only 13 days. The instability of G6PD Mediterranean is even more pronounced, with a half-life measured in hours. The clinical correlation of this age related eznyme instability is that hemolysis in patient with G6PDA$^-$ is mild and is limited to the older deficient erythrocytes. In contrast, the enzymatic defect in G6PD Mediterranean is caused by a much greater enzyme instability, and RBCs of all ages are grossly deficient. Consequently, the entire RBC population of people with G6PD Mediterranean is susceptible to oxidant-induced injury, and this can lead to severe hemolytic anemia.

G6PD deficient erythrocytes exposed to oxidants (infection, drugs, fava beans) become depleted of GSH. This reaction is central to the cell injury in the disorders because once GSH is depleted there is further oxidation of other RBC sulfhydryl-containing proteins. Oxidation of the sulfhydryl groups on hemoglobin leads to formation of denatured globin or sulfhemoglobin. The latter form insoluble masses that attach to the red cell membrane, possibly by disulfide bridges, and these are known as Heinz bodies. In addition to hemoglobin oxidation, the direct oxidation of membrane sulfhydryl groups leads to the accumulation of membrane polypeptide aggregates, presumably because of disulfide bond formation between spectrin dimers and between spectrin and other membrane proteins. The result of these changes is the production of rigid, nondeformable erythrocytes that are susceptible to stagnation and destruction by reticuloendothelial macrophages in spleen and liver. Although hemolysis is mainly extravascular, intravascular hemolysis also occurs, giving rise to hemoglobinemia and hemoglobinuria. It should also be rememberred that some patients with unstable hemoglobinopathies also may manifest oxidant injury because these abnormal hemoglobin are inordinately susceptible to mild oxidant stress. In the vast majority of cases, however, when hemolysis occurs with oxidant injury it is caused by G6PD deficiency.

Hematologic and Clinical Features

The clinical expression of G6PD variants encompasses a continuous spectrum of hemolytic syndromes. In most affected people the deficiency state goes unrecognized, but in some it causes episodes or chronic anemia. Four clinical states are recognized (1) acute hemolytic anemia, (2) congenital nonspherocytic hemolytic anemia, (3) neonatal hyperbilirubinemia, and (4) favism.

Acute Hemolytic Anemia (Fig. 8.9)

With the most prevalent G6PD variants (G6PD A and G6PD Mediterranean), severe hemolysis occurs only after exposure to certain offending agents. The steady state is associated with no anemia or alteration in blood morphology, although a modest shortening of red cells survival can be demonstrated by using isotopic methods. Sudden destruction of the older, more deficient erythrocytes is triggered by drugs having a high redox potential and by selected infections or metabolic

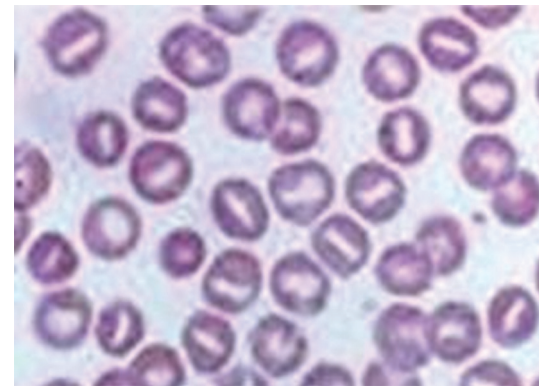


Fig. 8.9: G6PD deficiency, peripheral smear picture

perturbations. A classical example is set by primaquine induced hemolysis. After 2–4 days of primaquine ingestion all the signs, symptoms and laboratory results characteristic of an acute and dark urine with or without abdominal and back pain, are sudden in onset. An abrupt decrement of 3–4 g/dL in hemoglobin concentration occurs. Cell fragments, microspherocytes and eccentrocytes or bite cells are found in peripheral smears. In response to anemia, red cell production increases; an increase in reticulocytes is apparent within 5 days and is maximal by 7–10 days after onset of hemolysis. Despite continued drug exposure, the acute hemolytic process ends spontaneously after about 1 week, and hemoglobin concentration thereafter return to normal levels. The anemia is self-limited because the older, vulnerable population of erythrocytes is replaced by younger RBCs with sufficient G6PD activity to withstand an oxidative assault. Although red cell survival remains shortened as long as use of the drug continues compensation by the erythroid marrow effectively abolishes the anemia in subject G6PDA$^-$. In contrast, the hemolysis occurring with the G6PD Mediterranean variants is more severe because this variant has a very short intraerythrocytic half-life, and thus a larger population of circulating erythrocytes is vulnerable to injury. Sequestration of damaged RBCs occurs equally in liver and spleen. Hemolytic crises occur in heterozygous female subjects as well as hemizygous male patient. In virtually all cases acute hemolytic episodes are caused by the administration of drugs, are associated with infection or rarely occur with diabetic acidosis.

Drug-induced Hemolysis

Primaquine is one of several drugs that can precipitate hemolysis. The common denominator of these drugs is their interaction with hemoglobin and oxygen, thus accelerating the intracellular formation of H_2O_2 and other oxidising radicals. Aspirin can be given to patients with class II and III G6PD variants.

Infection-induced Hemolysis

Infection is probably the most common factor inciting hemolysis. About 20% of G6PD deficient subjects with pneumonia experience an abrupt drop in hemoglobin concentration. Various infectious agents implicated are: *Salmonella, Escherichia coli,* β-hemolytic streptococci and *Rickettsiae.* Hemolysis is particularly prominent in G6PD deficient subjects with viral hepatitis. The accelerated destruction of red cells imposes additional bilirubin load on an already damaged liver, resulting in an exaggerated increase in serum bilirubin level. Despite the magnitude of bilirubin retention, however, convalescence is generally complete and uneventful. Although hemolysis triggered by infection characteristically is mild, on rare occasion acute renal failure secondary to massive intravascular hemolysis has been observed. The exact mechanism for destruction of G6PD-deficient red cells during infection is not known. A possible explanation for this relationship between infection and hemolysis is that oxidant generated by phagocytosing macrophages diffuses into extracellular medium where they pose an oxidative threat to G6PD deficient erythrocytes.

Drugs and chemicals unsafe for class I, II and III G6PD variants	
Acetanilid	Primaquine
Furazolidone (Furoxone)	Sulfacetamide
Methylene blue	Sulfamethoxazole
Nalidixic acid	Sulfanilamide

Contd.

Drugs and chemicals unsafe for class I, II and III G6PD variants	
Naphthlene (Moth balls)	Sulfapyridine
Nitrofurantion (Furadantin)	Thiazolsulfone
Phenazopyridine (Pyridium)	Toluidine blue
Phenylhydrazine	Trinitrotoluene (TNT)

Drugs and chemical safe for class II and III G6PD variants	
Acetaminophen	Probenecid
Ascorbic acid	Procainamide
Aspirin	Pyrimethamine
Chloramphenicol	Quinidine
Chlorquine	Streptomycin
Colchicine	Sulfamethoxypyridazine
Diphenhydramine	Sulfisoxazole
Isoniazid	Trimethoprim
Menadione sodium bisulfite	Tripelennamine
Phenacetin	Vitamin K
Phenylbutazone	
Phenytoin	

Hemolysis Induced by Diabetic Acidosis

Diabetic ketoacidosis can trigger destruction of G6PD deficient red cells. Correction of acidosis and restoration of glucose homeostasis reverses the hemolytic process. Changes in pH, glucose and pyruvate have been proposed as possible mechanisms for hemolysis. Also, occult infection may be a common trigger for inducing both acute hemolysis and diabetic acidosis.

Congenital Nonspherocytic Hemolytic Anemia

With some G6PD variants, the deficiency of enzyme activity is sufficiently great that life-long hemolysis occurs in the absence of infection or drug exposure. These class I variant enzymes are extremely heterogeneous with respect to biochemical kinetics, but have in common low *in vitro* activity and marked instability. Most of these variants have DNA mutations at the glucose-6-phosphate or NADP binding sites. The hemolytic anemia associated with class I variants is indistinguishable from the congenital nonspherocytic hemolytic syndromes related to glycolytic enzyme deficiencies.

Anemia and jaundice often are noted first in the newborn period. Hyperbilirubinemia often necessitates exchange transfusion. Typically hemolysis occurs in the absence of a recognized triggering factor, although exposure to drugs or chemicals with oxidant potential exaggerates an already established hemolytic process. Beyond infancy, signs and symptoms of the hemolytic disorder are subtle and inconstant. Pallor is observed infrequently, scleral icterus is noted intermittently, and rarely the spleen is enlarged. The course may be complicated by parvovirus-induced aplastic crises. A temporary arrest of erythropoiesis, usually associated with a febrile illness, is attended by an abrupt drop in hemoglobin concentration. Often such a crises is the event that first leads to examination of blood. Exaggeration of anemia occurs after exposure to drugs with oxidant properties, even those that are safe for patients with class II and III G6PD variants. Hemolysis also can be accelerated with exposure to fava beans.

No hematologic atterations of the class I variants are distinctive. The hemolytic process may be fully compensated, although mild-to-moderate anemia is the rule (Hb 8–10 g/dL). Under basal conditions the usual reticulocyte count is 10–15%. Splenectomy generally is of little use.

In a few rare instances, leukocyte dysfunction caused by G6PD deficiency patients has been described in severely deficient patients. The abnormality is characterized functionally by defective bactericidal activity (but normal chemotaxis and phagocytosis), and clinically by recurrent infections with catalase-positive organisms. Overall, however, clinical infections are not a major problem in G6PD.

Neonatal Hyperbilirubinemia

Few G6PD variants that cause acute hemolysis also are associated with hyperbilirubinemia in the newborn period. At particular risk are neonates with the rare class I G6PD variants. From a practical perspective, however, neonatal hyperbilirubinemia is seen with the more common G6PD Mediterranean (class II). G6PD Canton may also be responsible. Untreated hyperbilirubinemia often leads to kernicterus with severe neurologic injury or death. The cause of hyperbilirubinemia in G6PD-deficient infants is not clear. The most common consideration is that it reflects increased bilirubin production caused by accelerated red cell breakdown. However, often no obvious external oxidant is responsible for red cell injury. The herbs used in China or clothing impregnated by naphthalene balls or drugs or chemicals taken by the mother in late gestation have been implicated as the inciting stimulus. However, because neonatal hyperbilirubinemia most commonly occurs in the absence of obvious exogenous oxidants, it has been suggested that hyperbilirubinemia may have another etiology, possibly related to impaired hepatic clearance of bilirubin. This hypothesis is supported by the observation that carboxyhemoglobin production, a marker of hemolysis or RBC breakdown, is the same in G6PD Mediterranean deficient neonates with and without hyperbilirubinemia. Also neonates with G6PD Mediterranean have been demonstrated to have a partial defect in bilirubin glucuronide conjugation, similar to that seen in Gilbert's disease. Taken together, these observations suggest that hyperbilirubinemia in these infants may be related to impaired liver clearance of bilirubin, although as yet no known role of G6PD is established in bilirubin conjugation.

Favism

The fava bean (*Vicia fava*) is toxic for G6PD deficient individuals (mostly associated with G6PD Mediterranean variants and hence usually encountered in Italy and Greece but few cases are also observed in Asia too). Most reported cases of favism result from ingestion of fresh beans with peak seasonal incidence of this disorders coinciding with harvesting of the bean (April and May). Hemolysis of comparable severity can follow consumption of dried beans. The syndrome has been observed in nursing infants of mothers who have eaten the beans, as well as in a newborn infant whose mother had eaten fava beans 5 days before delivery. Moreover, inhalation of pollen from the fava plant has also been incriminated.

Favism occurs most commonly in children between ages of 1 and 5 years. Like other G6PD deficiency syndromes, it occurs primarily in males. Symptoms of acute intavascular hemolysis occur within 5–24 hours of ingestion of the bean. Headache, nausea, back pain, chills and fever are followed by hemoglobinuria, anemia and jaundice. The drop in hemoglobin concentration is precipitous and often severe. In the absence of transfusion of the anemia can be fatal.

Favism does not occur in all susceptible G6PD people and it is thought that an additional genetic factor is involved, presumably related to how fava beans oxidants are metabolized. A factor other than enzyme deficiency is operative. A decrease in the urinary excretion of D-glucaric acid and impaired formation of salicylamide glucuronide in subjects with favism have been interpreted to reflect an abnormality in the hepatic conjugation of glucuronide and, by inference, are related to the toxicity of some factor derived from fava beans. Two pyrimidine aglycons, divicine and isouramil, have been implicated as the toxic components of fava beans. Both compounds rapidly overwhelm the GSH-generating capacity of G6PD-deficient cells and reproduce many of the metabolic derangements noted during hemolytic episodes. Till date, however, there are no convincing data to explain the erratic hemolytic episodes in favism.

Diagnosis

Most commonly, anemia is first recognized during or after an infectious illness or after exposure to one of several suspect drugs or chemicals. Clinical and hematologic findings reflect the severity of hemolysis but are not themselves results of G6PD deficiency. Irregularly contracted erythrocytes (eccentrocytes with hemoglobin puddled to one side of RBC) and bite cells are seen in the Wright–Stained peripheral blood smear. Earlier, these bite cells were considered a consequence of splenic removal of Heinz bodies. Now, however, it is understood that these RBCs contain a coagulum of hemoglobin that has separated from the membrane, often leaving an unstained nonhemoglobin-containing cell membrane (creating the appearance of a bite removed from the cell). These morphologic alterations are a consequence of the oxidative assaults on hemoglobin. Brilliant cresyl blue supravital stains of the peripheral blood may reveal Heinz bodies during hemolytic episodes.

The specific diagnosis of G6PD deficiency is made by adding a known amount of hemolysate to an assay mixture containing substrate (glucose-6-phosphate) and cofactor (NADP) and then spectrophotometrically measuring the rate of NADPH generation. Alternatively a variety of screening tests that use hemolysate as a source of enzyme can also be used. The fluorescent spot test is the simplest, most reliable, and most sensitive screening method. This test is based on the fluorescence of NADPH indirectly by measuring the transfer of hydrogen ions from NADH to an acceptor. In the methemoglobin reduction test (MRT), methylene blue is the acceptor used for the transfer of hydrogen from NADPH to methemoglobin, thereby facilitating its reduction. It is important to mention this test because, when combined with a technique for the elution of methemoglobin from intact cells, it can be used to detect relative G6PD sufficiency of individual RBC, thereby detecting the carrier state with approximately 75% reliability. Regardless of the specific test used to detect G6PD deficiency, however, false-negative reactions occur if the most severely enzyme-deficient RBCs have been removed by hemolysis. In such cases the family members should be studied. Another approach is to wait until the hemolytic crisis is over and reevaluate the patient after the RBC mass is repopulated with cells of all ages (approximately 2–3 months).

Treatment

G6PD deficiency management is determined by the syndrome with which it is associated. Acute significant fall of hemoglobin requires RBC transfusion. This is the case more commonly in G6PD Mediterranean (class II) than in G6PDA^{-} (Class III). All affected individuals should avoid exposure to drugs known to triggger hemolysis. Pregnant and nursing women known to be heterozygous for the deficiency should avoid ingestion of

drugs with oxidant potential because some gain access to the fetal circulation and breast milk. If indication for its use is sound, however, an offending drug may be given despite modest shortening of red cell survival. For example, primaquine is safely given to patients with the G6PDA$^-$ variants, provided it is started cautiously (15 mg/day or 45 mg once or twice weekly) and the blood count is monitored closely. The mild anemia caused by its administration is rapidly corrected by a compsensatory erythropoietic effect and does not recur unless the dose of drug is enhanced. Transfusion therapy is unnecessary unless hemolytic episodes are compounded by a concurrent arrest of erythropoiesis.

Chronic nonspherocytic hemolytic anemia is caused by class I G6PD variants may need more active intervention. Exchange transfusion during the first week of life often is needed to prevent bilirubin encephalopathy. Beyond the newborn period, anemia persists, but it rarely is severe enough to require blood transfusion. During aplastic crisis, however, transfusion may be lifesaving. As with other syndromes resulting from G6PD deficiency, drugs capable of exaggerating hemolysis be avoided. Splenectomy occasionally brings about a modest improvement in hemoglobin concentration, but it is generally without benefit. Because of the antioxidant properties, vitamin E has been proposed as a therapeutic agent, but subsequent evaluation of large doses of the vitamin failed to demonstrate an ameliorative effect on anemia.

Screening

Blood bank screening of donors should be conducted in areas where G6PD deficiency (presumably class II variants) is common. The recommendation currently is not a standard blood bank procedure. Screening for public at large is unwarranted.

RELATED DISORDERS OF HMP SHUNT AND GLUTATHIONE METABOLISM

In addition to G6PD, other enzymes of the HMP shunt pathway (6-phosphogluconate dehydrogenase), the closely linked reactions of glutathione metabolism (glutathione reductase, glutathione peroxidase), and glutathione synthetic pathway are important in protecting RBC against oxidant injury. Rare abnormalities in several of these enzymes have been reported, and in some cases are associated with hemolysis.

6-Phosphogluconate Dehydrogenase Deficiency

Although 6PGD deficiency is well-documented, it appears to have little or no significance for red cell viability. Presumably this reflects the fact that NADPH is generated by the proximal enzyme G6PD, suggesting that the second dehydrogenase may not be necessary for cell integrity.

Glutathione Reductase Deficiency

Oxidized glutathione (GSSG) is reduced in the presence of NADPH by glutathione reductase (GSSG-R). The enzyme contains flavin as one of its components, so enzyme activity depends on the dietary availability of riboflavin. Not surprisingly partial GSSG-R deficiency is a common feature of disorders that are compounded by suboptimal nutrition. GSSG-R-levels are restored within days by the administration of physiologic quantities of riboflavin. The association between riboflavin-induced GSSG-R deficiency and various disease states is of no hematologic consequence.

Glutathione Peroxidase Deficiency

Glutathione peroxidase (GSH-Px) catalyzes the oxidation of reduced glutathione (GSH) by peroxides, including hydrogen peroxide and organic hyperoxides. Rare cases of hemolysis

in association with moderate deficiency of RBC GSH-Px activity have been described in adults and children. However, the general consensus is that GSH-Px deficiency is probably not a cause of hemolysis or other hematologic problems.

Levels of GSH-Px in the erythrocytes of healthy newborn infants are approximately half those seen in the older children and adults. Adult levels are achieved at 6–10 months of age. GSH-Px deficiency has been incriminated as a cause for neonatal jaundice, although a relationship between GSH-Px levels and magnitude of bilirubinemia has not been identified in prospective studies. When cord RBCs are stressed with peroxide under conditions preventing regeneration of GSH, the amount of GSH oxidised is similar for cord and adult erythrocytes. Low GSH-Px activity in cord RBC thus appears to have no functional significance.

Defects in Glutathione Synthesis

Glutathione is actively synthesized in RBC and has an intracellular half-life of only 4 days, in part because of cellular efflux of oxidized glutathione (GSSG). RBCs are capable of *de novo* GSH synthesis, and this is accomplished by two critical enzymes. γ-glutamyl-cysteine synthetase (γ-GC Synth) catalyses the first step in GSH synthesis, the formation of γ-glutamyl-cysteine from glutamic acid and cysteine. Glutathione synthetase (GSH synth) catalyzes the formation of GSH from glutamyl-cysteine and glycine. In many tissues but not RBCs, these two enzymes are part of the γ-glutamyl cycle, which is involved with the synthesis and degradation of GSH and also thought to have a role in amino acid transport across the cell membranes. Hereditary hemolytic anemia, characterized by reduced GSH content, has been reported in patients with deficiencies of both γ-GCSynth and GSHSynth activity. The clinical effect of these disorders depends on the severity of enzyme deficiency and whether the γ-glutamyl cycle also is affected in nonerythroid tissues.

γ-glutamyl-cysteine Sysnthetase (γ-GCSynth) Deficiency

γ-GCSynth deficiency is a rare hemolytic anemia. The disorder appears to have an autosomal recessive inheritance. In homozygous state 95% of γ-CGSynth RBC activity is lost and leads to hemolytic anemia. γ-GCSynth deficiencies may be associated with hemolytic anemia, mild intermittent jaundice, cholelithiasis, and splenomegaly. They may also suffer from severe neurologic dysfunction and generalized aminoaciduria.

Glutathione Synthetase Deficiency

GSH synthetase deficiency has been incriminated as the cause of chronic hemolytic anemia alone (caused by isolated RBC enzymes deficiency) and as the cause of a generalized syndrome (caused by enzyme deficiency in many tissues) characterized by mild hemolytic disease, severe metabolic acidosis, and mental deterioration. The first syndrome, mild hemolytic anemia and intermettent jaundice has been described in several families. Oxidant drugs and fava beans enhance hemolysis.

Splenomegaly has been noted in about 50% of cases. A concurrent deficiency of glutathione-S-transferase is thought to be caused by the instability of this enzyme in the absence of adequate intracellular GSH. The second more generalized syndrome is characterized by mild hemolytic anemia, persistent metabolic acidosis presenting in the newborn period, and progressive cerebral and cerebellar degeneration. The latter is characterized pathologically by atrophy of the granular cell layer of the cerebellum and focal lesions in the cortex and thalamus. Acidosis is caused by the accumulation of 5-oxoproline, a metabolic product of γ-glutamyl-cysteine. Abnormally large quantities of the dipeptide are produced because of the loss of feedback inhibition of

γ-glutamyl-cysteine synthetase by GSH. The disorder is recently suspected in patients with hemolytic anemia and markedly reduced RBC GSH content. Virtually no-GSH synth activity is detected in homozygous deficient patients. Rarely, therapy is required for the hematologic consequences of GSH deficiency. Exposure to drugs and chemicals with oxidant potential should be avoided by patients with chronic hemolytic anemia.

Glutathione S-transferase Deficiency

Glutathione S-transferase (GSH-ST) conjugates GSH with electrophilic xenobiotics to detoxify them. The enzyme may play a role in the active transport of GSH-xenobiotic conjugates from the red cell. A single case of GSH-ST deficiency with mild hemolytic anemia is known. The significance of this enzyme deficiency remains to be defined.

HEREDITARY HEMOLYTIC ANEMIAS ASSOCIATED WITH ABNORMALITIES OF ERYTHROCYTE GLYCOLYSIS AND NUCLEOTIDE METABOLISM

RBC enzymopathies can cause just hemolysis or be associated with multisystem disorders. Some enzymopathies have no adverse effects on RBC function, or if they occur in patients with hemolytic anemia, it is not always clear that the enzyme deficiency and hemolysis are causally related.

General Consideration (Table 8.4)

A mature red blood cell is anucleate (therefore, it cannot divide), lacks ribosomes (hence, cannot synthesize proteins) and it also does not possess mitochondria (thereby incapable

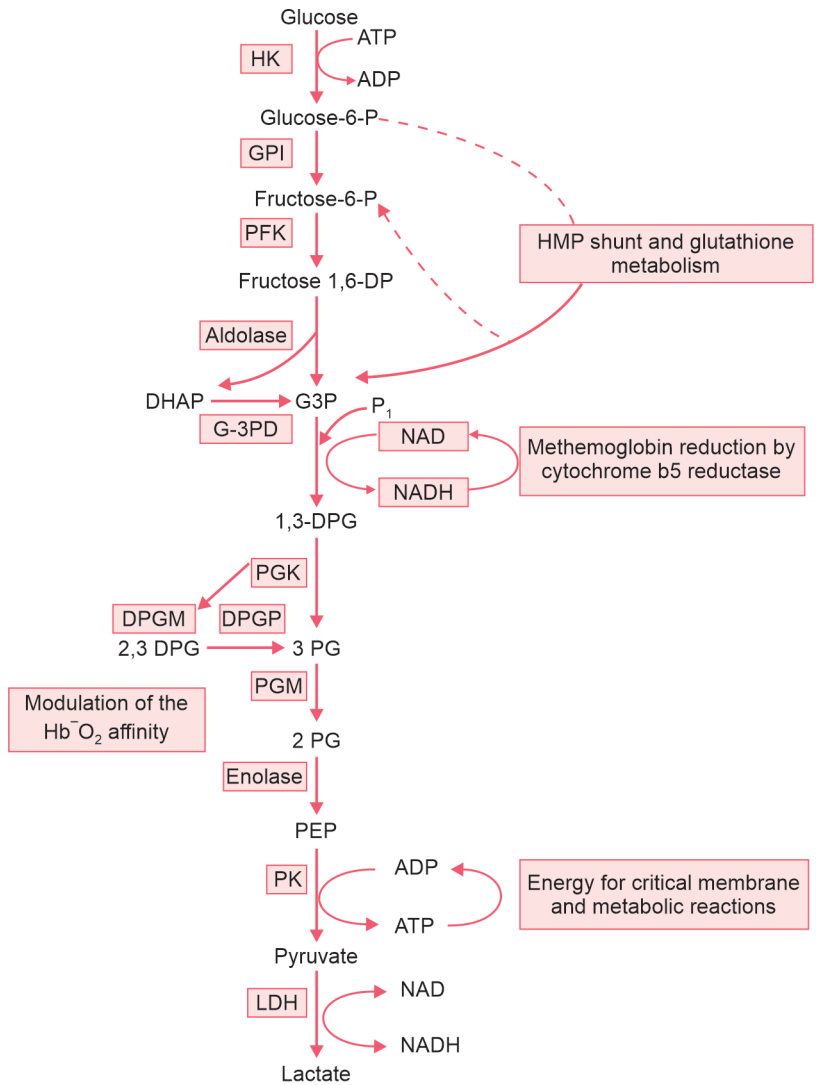
Table 8.4: General considerations—enzymopathies (glycolytic)

Enzymopathy	*Approximate % of enzymopathies*	*Mode of inheritance*	*Molecular abnormalities known*	*Effects of enzymopathy*
Hexokinase (HK)	<1	AR	2	Mild/severe CNSHA
Glucose phosphate isomerase (GPI)	3–5	AR	12	Moderate/severe CNSHA
Phosphofructokinase (PFK)	<1	AR	13	Mild CNSHA +/– myopathy
Aldolase	<1	AR	1	Mild/moderate CNSHA ?hepatomegaly, psychomotor retardation
Triosephosphate isomerase (TPI)	<1	AR	5	Moderate/severe CNSHA
Phosphoglycerate kinase (PGK)	<1	X-linked	5	Mild/severe CNSHA, psychomotor retardation, seizures, extrapyramidal disease
Pyruvate kinase (PK)	80–90	AR	47	Moderate/severe CNSHA
Pyrimidine 5′ nucleotidase (P5′N)	2–3	AR		Moderate CNSHA
Adenosine deaminase (ADA) excess	<1	AD	—	Mild CNSHA
Adenylate kinase (AK)	<1	AR	—	(?) CNSHA

AR = autosomal recessive, AD = autosomal dominant CNSHA = Chronic nonspherocytic hemolytic anemia

of oxidative phosphorylation). Glucose is the main metabolic substrate of RBCs and it is metabolized by two major pathways, the glycolytic or "energy producing" pathway and the hexose monophosphate (HMP) shunt or "protective" pathway. Under normal conditions approximately 90% of glucose flows through glycolysis, with a much smaller fraction being channeled through the HMP pathways (Flowchat 8.4). However, the fraction of glucose entering the pentose phosphate pathway can increase significantly under increased oxidative stress. The major products of glycolysis are adenosine triphosphate (ATP, a source of energy for numerous RBC membrane and metabolic reactions), nicotinamide

Flowchart 8.4: Overall metabolic pathway of glycolysis in the RBC



HK-hexokinase, GPI = glucose phosphate isomerase, PFK = phosphofructokinase, TPI = triosephosphate isomerase, G3PD = glyceraldehyde 3-phosphate dehydrogenase, PGK = Phosphoglycerate kinase, 2–3 DPGM = 2,3 diphosphoglycerate mutase, 2,3 DPGP = 2,3 diphosphoglycerate phosphatase, PGM = Phosphoglycerate mutase, PK = phosphokinase LDH = lactate dehydrogense 1–3 DPG = 1,3 phosphoglycerate, G3P = glyceraldehyde 3 phosphate, DHAP = dihydroxyacetone phosphate, 3, PG = 3, Phosphoglycerate 2-PG = 2, Phosphoglycerate PEP = phosphoenolpyruvate

adeninedinucleotide (NADH, a necessary cofactor for methemoglobin reduction by cytochrome b5 reductase), and 2,3-DPG (an important intermediate that modulates hemoglobin-oxygen affinity). The cause of hemolysis in RBC glycolytic defects is thought to be abnormal membrane function, but the precise relationship between decreased ATP production, abnormal accumulation other glycolytic intermediates, and impaired membrane function leading to red cell destruction is not known.

Hemolytic anemias caused by glycolytic enzymopathies are rare, affecting only a few thousand patients, unlike G6PD (glucose-6-phosphate dehydrogenase) deficiency, which affects millions worldwide. Abnormalities in virtually every glycolytic enzyme have been described, although over 90% of cases associated with hemolysis are caused by pyruvate kinase (PK) deficiency. Most glycolytic enzymopathies manifest an autosomal recessive pattern of inheritance. Heterozygotes almost always are hematologically normal although their RBCs contain lower than normal levels of enzyme activity

Clinical manifestations of hemolysis caused by glycolytic enzymopathies include chronic anemia, reticulocytosis, and some degree of hyperbilirubinemia. In the vast majority of cases the hemolytic process is recognized and diagnosed in childhood. After there is a history of neonatal jaundice, usually requiring an exchange transfusion, and rarely causing kernicterus. The degree of chronic anemia varies, usually with infection, because of accelerated hemolysis or transient aplastic crises associated with parvovirus infection. In some enzymopathies, such as PK deficiency, the adverse effects are restricted to the RBC. In other cases, such as PFK, TPI, and PGK deficiencies, hemolytic anemia is but one feature of multisystem disease. The possibility of a glycolytic defect usually is considered when anemia cannot be explained by the more common causes of chronic hemolysis (i.e. hereditary spherocytosis and hemoglobinopathies).

There are no specific morphologic abnormalities, although anisocytosis and poikilocytosis are common. In virtually all cases a specific assay of RBC enzyme activity is necessary to establish diagnosis. Screening tests are available, but these are of limited value. When there is a partial deficiency or a mixed population of RBC. An important problem in identifying glycolytic enzymopathies relates to the fact that the most severely deficient cells are removed (i.e. hemolyzed) and the cells remaining may be biochemicaly less abnormal. Consequently, false negative assays can obscure the correct diagnosis. For this reason, it sometimes is helpful to study parents and other family members for the presumed heterozygous state of enzyme deficiency. In some cases DNA based molecular diagnostic tests may be available.

Treatment of RBC glycolytic defects is similar to that of other chronic hemolytic anemias. RBC transfusions are often indicated. Splenectomy often is beneficial in severely anemic patients because the spleen (along with the liver) participates in the destructions of enzymatically abnormal cells. In most cases, however, the response to splenectomy is only partial, and hemolysis usually continues although RBC transfusion requirements may decrease. Cholelithiasis is a common problem and all patients with glycolytic enzymopathies should have periodic gallbladder ultrasound examinations, even postsplenectomy.

PYRUVATE KINASE DEFICIENCY

Of the glycolytic enzymopathies, PK deficiency is the most common. Most cases are reported from Europe, USA and Japan.

Biochemical Genetics

PK catalyzes the conversion of phosphoenol-pyruvate (PEP) to pyruvate; this is one of the two glycolytic reactions resulting in net ATP production. The enzyme is allosterically activated by fructose-1,6-diphosphate (FDP) and is inhibited by its product, adenosine triphosphate (ATP). Pyruvate kinase is a tetrameric protein with a molecular weight of 230 kDa. There are two PK genes (PKM2 and PKLR) that encode for four distinct PK isoenzymes. The hemolytic anemia associated with PK deficiency is caused by mutation of the PKLR gene. During normal erythroid differentiation, the PK isoenzyme switches from the M2 to the R type. Almost 50 different mutation of the PKLR gene have been identified as causes of chronic hemolytic anemia. Most of these are missense mutation although a few are either nonsense or insertional mutations, deletions, or splicing abnormalities. Most PK variants are abnormal proteins that differ with respect to their biochemical kinetics and physical properties.

Pathophysiology

PK deficiency results with impaired glucose use and, thereby, decreased pyruvate and lactate production. In addition, glycolytic intermediates proximal to the block accumulate in red cells, and the levels of 2,3 diphosphoglycerate (2,3-DPG) may increase up to three-fold. The major impairment caused by PK deficiency is a reduced capacity to generate ATP. Paradoxically, however, PK deficient patients with high reticulocyte counts may have normal or even elevated levels of ATP. This occurs because PK-deficient reticulocytes generate ATP through mitochondrial oxidative phosphorylation and this is associated with a six to seven-fold increase in oxygen consumption compared to normal reticulocytes. The advantage of oxidative phosphorylation for PK-deficient reticulocytes is that ATP can be generated requiring minimal flow of pyruvate from glycolysis. When reticulocytes mature, however, mitochondria disappear, oxidative phosphorylation ceases, and ATP levels fall. The effects of this vary because there are marked cellular differences in the enzyme content of PK-deficient red cells. In severely PK-deficient RBC, the fall in ATP leads to cell injury although the precise ATP-dependent reactions leading to irreversible membrane injury are not known. Irrespective of the mechanism, the result of the changes is a loss of membrane plasticity and the formation of a rigid RBC marked for premature destruction in the spleen or in the liver after escape from the spleen.

The reticulocytes in PK deficiency have greater adhesive tendencies and endure a longer sojourn in the spleen, where limited oxygen and glucose restrict effective oxidative phosphorylation. Impaired ATP production then leads to RBC destruction in the spleen or liver after escape from the spleen. An exaggerated and sustained reticulocytosis follows after splenectomy. As has been discussed earlier 2,3 DPG may be upto three times normal in PK deficient subjects. This increase is responsible for a rightward shift in the oxygen dissociation curve of hemoglobin. Therefore, PK deficient individuals have greater exercise tolerance than would be expected from the degree of anemia.

Clinical Features

Clinical features of PK deficiency are quite variable, ranging from pronounced neonatal jaundice requiring multiple exchange trasnfusions and occasionally complicated by kernicterus, to a fully compensated hemolytic process detected as an incidental finding in adults. The PK deficiency associated jaundice is accompanied with anemia and splenomegaly. Beyond the neonatal period, anemia of varying degree, jaundice and splenomegaly characterize erythrocyte PK deficiency. Here, anemia may be well-tolerated

because of the increased red cell 2,3-DPG content which is responsible for a rightward shift in the oxygen dissociation curve. The clinical course may be complicated by aplastic crises, characterized by an abrupt temporary arrest of erythropoiesis and a precipitous drop in hemoglobin concentration and reticulocyte count. These crises are usually related to infection with a parvovirus-like agent that is cytotoxic for erythroid progenitors. After the first decade of life, gallstones are detected with increasing frequency. Bone changes associated with hyperplastic bone marrow, such as those seen in thalassemia occasionally may lead to frontal bossing. Chronic leg ulcers occur rarely. Pregnancy has been tolerated without unusual complications. Iron overload is a predictable complication of chronic transfusion therapy, but it may occur also in patients with a limited or no history of transfusions (this may be because of coexisting inheritance of the more common hereditary hemochromatosis gene).

Diagnosis

Anemia caused by PK deficiency is moderate to severe in degree (Hb being 6–12 g/dL). The peripheral smear shows morphologic hallmarks of accelerated erythropoiesis: Polychromatophilia, anisopoikilocytosis, and variable numbers of nucleated red cells. Irregularly contracted erythrocytes with surface spicules, tailed poikilocytes, and acanthocytes have been found in smears of some affected patients. Post-splenectomy, reticulocytosis may further enhance (because of long survival of PK deficient reticulocytes following splenectomy).

In cases where PK deficiency is suspected, a direct quantitative assay of the enzyme is essential. WBCs must be excluded because PK activity of WBCs is 300 times that of normal red blood cells. Molecular diagnostic techniques that require small amounts of DNA are very useful. Families with a PK deficient child must go in for parental diagnosis too. DNA analysis is the test of choice as biochemical investigations require large volumes of blood.

Acquired erythrocyte PK deficiency has been observed in patients with acute leukemia, preleukemia, and refractory sideroblastic anemia and exposure to chemotherapy. Reasons may be (a) injury of bone marrow stem cells with disturbance of protein synthesis or (b) a post-translational modification of the enzyme. In acquired PK deficiency cases the degree of enzyme deficiency is not severe enough to cause hemolytic anemia.

Treatment

During the first year of life, severe anemia is managed with red cell transfusion. Maintenance of the hemoglobin concentration above 8 g/dL permits normal growth and development while minimising the risk of life-threatening aplastic crises. The decision for transfusion therapy must be based on patient tolerance of anemia rather than an arbitrary level of hemoglobin. Because of increased red cell 2,3-DPG content, patients may tolerate moderately severe anemia with little effects.

Splenectomy is useful for long-term control of anemia. However, because of the well-known risk of post-splenectomy sepsis by *Streptococcus pneumoniae* in young children, surgery should be delayed until 5 years of age whenever possible. Splenectomy is followed by an improvement but not correction of the hemolytic disorders. Transfusion requirements, if present before splenectomy, decrease or are eleminated. In all patients an incompletely compensated hemolytic process with reticulocytosis persists. Some degree of hyperbilirubinemia and a risk for gallstone formation usually persists. Bone marrow transplantation may be considered in future.

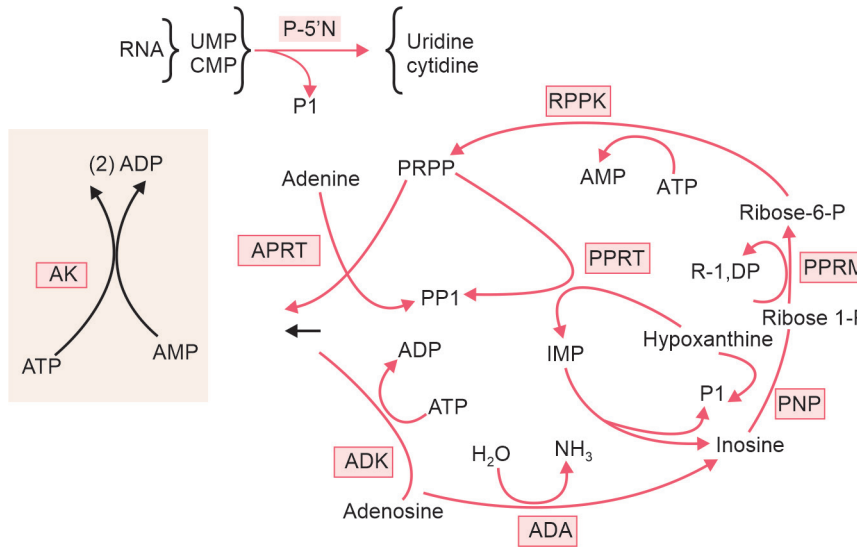
ABNORMALITIES OF PURINE AND PYRIMIDINE NUCLEOTIDE METABOLISM (FLOWCHART 8.5)

In certain immune and metabolic disorders caused by inborn errors of purine metabolism, erythrocytes share the same enzymatic deficiency, without any adverse effect on RBC function or viability. The RBC enzyme abnormalities with these cases serve as marker of disease in other tissues. Such disorders include adenosine deaminase (ADA) deficiency associated with severe combined immune deficiency, nucleoside phosphorylase deficiency associated with impaired T-cell immunity, complete hypoxanthine guanine phosphoribosyl transferase (HGPRT) deficiency associated with Lesch-Nyhan syndrome, partial HGPRT deficiency (30% normal activity) associated with increased uric acid production and gouty arthritis, elevated ribosephosphate pyrophos-phokinase (PRPP synthetase) activity associated with increased urate production and gout and adenine phosphoribosyl transferase (APPRT) deficiency associated with renal stone formation.

Pyrimidine 5' Nucleotidase Deficiency

Ribosomal RNA in normal reticulocytes is degraded to 5'nucleotides. The enzyme P5'N further catalyzes the degradation of cytidine and unidine mononucleotides to inorganic phosphate and the corresponding nucleoside. Reticulocytes deficient in P5'N accumulate large quantities of cytidine and uridine compounds, increasing the total nucleotide pool to more than five times that present in normal red cells. In addition to the increased nucleotide pool content, elevated levels of reduced glutathione and decreased activity of ribosephosphate pyrophosphate synthetase (PRPP) are consistent but unexplained findings of P5'N deficiency. As a consequence of impaired ribosomal degradation, intracellular aggregates form in P5'N deficient cells, and these appear as basophilic stippling on Wright-Stained peripheral blood smears. P5'N deficiency is very rare and the disorder follows an autosomal recessive mode of transmission. Homozygotes have life-long hemolytic anemia

Flowchart 8.5: Scheme of purine and pyrimidine metabolism in mature RBCs



ADA = Adenosine deaminase, ADK = Adenosine kinase, AK = Adenylate kinase, APRT = Adenine phosphoribosyltransferase, RPPK = Ribopyrophosphoryl kinase, PPRM = Pyrophosphoribosyl mutase, PPRT = Pyrophosphoribosyl transferase, PNP = Purine nucleoside phosphorylase, P5'N = Pyrimidine 5' nucleotidase AMP = Adenosine monophosphate, IMP = Inosine monophosphate.

associated with splenomegaly and intermittent jaundice. The disorder is characterized by mild to moderate anemia, reticulocytosis and hyperbilirubinemia. Splenectomy is followed by a moderate increase in hemoglobin concentration but affords no significant benefits. There are no P5′N abnormalities in platelets or leukocytes of affected patients. Development retardation has been noted. The possibility of P5′N deficiency as the cause of hemolysis is suggested by the presence of marked basophilic stippling, a unique RBC morphologic feature characteristic of the hemolytic disorder. Definitive diagnosis needs demonstration of decreased RBC nucleotidase activity or increased pyrimidine nucleotides in erythrocytes. Normally RBC nucleotides are almost entirely adenine nucleotides. However, in P5′N deficiency, up to 80% of the nucleotide pool may be pyrimidine nucleotides. The latter is the basis for a simple screening test that uses ultraviolet spectroscopy to demonstrate a shift in the absorption spectrum of red cell lysates.

The exact mechanism as to why P5′N deficiency leads to hemolytic anemia is not known, although there are a variety of proposed mechanisms. One curious explanation relates to a concomitant decrease in the G6PD activity of P5′N deficient cells, which is associated with decreased pentose phosphate shunt activity and increased Heinz body formation. The mechanism whereby P5′N deficiency affects pentose phos-phate shunt activity involves both competitive inhibition of glucose-6-phosphate and noncompetitive inhibition NADP for G6PD by pyrimide nucleotides. Interestingly, both P5′N and G6PD deficiencies are characterized by hemolytic crises complicating intercurrent infections.

P5′N enzyme is readily inactivated by heavy metals such as lead, and it has been proposed that the basophilic stippling in lead poisoning is secondary to acquired P5′N deficiency.

Adenosine Deaminase Excess

Adenosine is a common substrate for two different enzymes, adenosine kinase (ADK) and adenosine deaminase (ADA). Normal metabolism is thought to proceed through ADK with phosphorylation of adenosine to form AMP. In the presence of plasma adenosine, ADK thereby helps maintain the red cell adenine nucleotides pool. ADA leads to the deamination of adenosine with the formation of inosine, and this enzyme is critical for normal immune function. Hereditary deficiency of ADA is associated with severe combined immunodeficiency and because the enzyme deficiency also exists in erythrocytes, assay of red cell ADA can be used to diagnose this immune disorders. Red cell adenine nucleotide content is increased in severe ADA deficiency, but this has no adverse effects on RBC, and there is no anemia surprisingly; however, hereditary hemolytic anemia occurs in association with a 60–100-fold excess of normal ADA activity, and this has been described in a few families. In patients with hemolysis and increased RBC-ADA activity, erythrocyte ATP content is reduced. The decrease in adenine nucleotides presumably occurs because elevated ADA activity effectively competes with ADK, producing a relative deficiency of the later enzyme.

This rare hemolytic enzymopathy is unique because it is associated with an enzyme excess and it is inherited in an autosomal dominant pattern. Clinical features include mild to moderate anemia, reticulocytosis, and hyperbilirubinemia. No other tissues share in enzyme excess, and there are no other systemic effects. There are no distinguishing clinical, hematologic, or morphologic features to aid in the diagnosis of this condition. The specific diagnosis can be suspected if red cell ATP levels are low and confirmed by demonstrating increased ADA activity with hemolysate.

Adenylate Kinase Deficiency

Adenylate kinase catalyses the interconversion of adenine nucleotides (AMP+ATP → 2ADP).This is thought to be the only enzyme reaction in mature RBC that can lead to ADP synthesis from AMP, and thus AK appears to have a critical role in salvaging AMP and protecting the erythrocyte adenosine nucleotide pool. In erythrocytes, AMP is formed in two reactions: the ADK-mediated phosphorylation of adenosine and the APRT-mediated phosphorylation of PRPP.

Hereditary nonspherocytic hemolytic anemia in association with AK deficiency has been described in a few cases. The limited data available suggest an autosomal recessive mode of inheritance of the enzyme deficiency. However, it is not entirely clear that this enzyme abnormality is causally related to hemolysis in these patients.

AUTOIMMUNE HEMOLYTIC ANEMIA (AIHA)

AIHA is used to describe a group of hemolytic anemias which result from the development of antibodies directed against antigens on the surface of the patient's own RBCs (act as autoantibodies). Usually of the IgG type, they can also be of IgM or IgA variety and some can bind complement. AIHA pathogenesis is not certain. The formation of autoantibodies may be due to a breakdown in T-cell regulation of B cells with emergence of a hostile clone of immunocytes, or to a change in the structure of an antigen on the patient's red cells which is then recognized as foreign by the immune system.

AIHA is a rare, but not an uncommon disorder and occurs in every grade of severity. Can be chronic mild asymptomatic to an acute rapidly fatal disease. AIHA can be classified: (a) As per the temperature at which the antibody reacts with the RBCs into warm antibody and cold antibody types; and (b) As per etiology into idiopathic and secondary. A detailed classification is given below.

CLASSIFICATION

According to Temperature at which Antibodies (Ab) React with RBCs (Table 8.5)

- Warm antibody
- Cold antibody

According to Etiology

- Primary (50%), more often in females
- Secondary (50%), more often in males.

Table 8.5: Comparison of warm and cold AIHA

	Warm AIHA	*Cold AIHA*
Optimal reactivity	>32°C	<30°C
Immunoglobulin class	IgG	IgM (exception: PCH-IgG)
Complement activation	May bind complement	Binds complement
Hemolysis	Usually extravascular (no cell lysis)	Usually intravascular (cell lysis)
Frequency	70–75% of cases	16% of cases (PCH: 1–2%)
Specificity	Frequently Rh	Ii system (PCH: antiP)

Warm-reactive Antibodies

a. Primary or idiopathic, without obvious cause
b. Secondary, associated with
   - Systemic lupus erythematosus and other autoimmune disorders, chronic active hepatitis, rheumatoid arthritis, ulcerative colitis, myasthenia gravis.
   - Chronic lymphocytic leukemia, lymphomas, etc.
   - Nonlymphoreticular tumors, carcinomas, ovarian teratoma.
   - Viral infections
   - Immune deficiency syndromes.

Cold-reactive Antibodies

a. Primary or idiopathic cold agglutinin disease
b. Secondary cold agglutinin disease associated with
   - Infection: *Mycoplasma pneumoniae,* infectious mononucleosus, and other viral infections
   - Chronic lymphocytic leukemia, lymphomas
c. Paroxysmal cold hemoglobinuria
   - Idiopathic
   - Secondary
     - Syphilis
     - Viral infections—measles, mumps.

Drug-induced Immune Hemolytic Anemia

a. Penicillin type
b. α-Methyldopa type
c. Stibophen (innocent bystander) type
d. Nonimmunologic protein adsorption

WARM ANTIBODY AIHA

Warm Antibody Autoimmune Acquired Hemolytic Anemia

- AIHA occurs at all ages, but adults are affected more frequently than children.
- Idiopathic type affects females more commonly than males, and usually occurs after the age of 40 years.
- Secondary form may complicate a number of diseases
  - Lymphocytic leukemia,
  - Malignant lymphomas, and
  - Systemic lupus erythematosus (SLE)
- Drugs-methyldopa

Clinical Picture

Usually—insidious onset with symptoms of anemia.

In most secondary cases—the underlying disease is obvious when the hemolysis develops, sometimes hemolytic anemia is the first symptom, and the clinical manifestations of the underlying disease may develop months or even year later. Mild-to-moderate jaundice is usual, but is absent in about 25% cases.

The spleen is nearly always palpable, but rarely extends below the umbilicus. A very large spleen suggests a chronic lymphocytic leukemia or malignant lymphoma. Even when the spleen is impalpable, it is inevitably enlarged. Moderate hepatomegaly is usual.

Lymph node enlargement does not occur in idiopathic cases, but is frequent in secondary cases. Hemoglobinuria is unusual, but occasionally occurs with an acute exacerbation of hemolysis. Hemosiderinuria is an occasional finding.

In young children, and in some adults, the onset may be sudden, secondary to a minor bacterial or viral infection. Anemia develops quickly, with jaundice and constitutional symptoms such as malaise, fever, headache, vomiting and pains in the abdomen and back. Intravascular hemolysis is evident, with hemoglobinemia and hemoglobinuria; sometimes oliguria and even anuria develop. The spleen becomes palpable. Hemolysis usually stops on its own after weeks or months and is followed by complete recovery. Rarely, death may occur from renal failure in severe cases. This form of transient acute warm antibody AIHA in children is usually idiopathic. In some cases, it is associated with cytomegalovirus infection.

Hematological Picture (Fig. 8.10)

The typical blood picture is of an anemia with reticulocytosis, spherocytosis, and a positive direct antiglobulin (Coombs') test.

The hemoglobin level differs markedly. In compensated cases it is within normal limits, while in very ill patients it may be 4 g/dL or less. A sudden drop to low values is the rule in acute cases.

Spherocytosis is usually present in the active disease, but may be less obvious in the silent phase. Spherocytosis is accompanied by an increase in the osmotic fragility of fresh blood, but in general the increase on incubation is less consistent than in hereditary spherocytosis. When spherocytosis is mild it may not be obvious in the blood film and is revealed only by the osmotic fragility test. A mild increase in MCV is usual.

Reticulocyte counts commonly range from 5–30%, but may be higher, and mild normoblastemia is frequent. Rarely, the reticulocyte count is normal or even reduced due to an aplastic crisis or folate deficiency. Polychromatic macrocytes are prominent in the film when the reticulocyte count is high and form a striking contrast to the microspherocytes. Erythrophagocytosis by monocytes may occasionally be observed.

The leukocyte count varies. In chronic cases of moderate severity, it is usually normal or, occasionally, moderately reduced. In acute cases or with severe hemolysis, leucocytosis is frequent, counts rising to 20–30 $\times 10^9$/L or even higher with a shift to the left.

The platelet count is usually normal, but occasionally lowered, sometimes sufficient to cause purpura. The serum bilirubin value usually ranges from 17 to 50 μmol/L, but is sometimes persistently normal despite continuing hemolysis.

The plasma haptoglobin level is reduced. The erythrocyte sedimentation rate is markedly increased in active states, but returns to normal during remissions. Immunoglobulin deficiency occurs in about 50% of cases. IgA deficiency is most common, although some patients show deficiencies of IgG and/or IgM, or of all three immunoglobulins; occasionally there is an excess of an immunoglobulin.

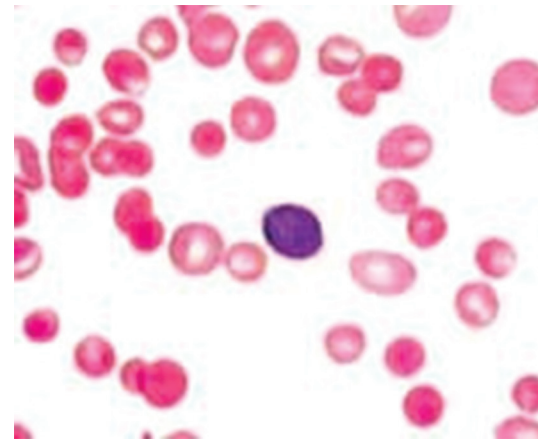


Fig. 8.10: Warm antibody AIHA, peripheral smear picture

Serum and red cell folate levels may be reduced. Blood drawn from routine examination often shows mild agglutination in the collection tube and on the blood film. This is not prevented by taking the blood into a warm syringe and keeping it at 37°C, and represents agglutination of red cells heavily coated with incomplete antibody. It should not be mistaken for the usually more intense agglutination of cold hemagglutinin disease.

Immunology

Autoantibodies may be shown *in vitro* in most cases of warm antibody AIHA. They are found on the red cell surface and also in the serum.

Antibodies on the Red Cell Surface

The presence of antibodies on the red cell surface is demonstrated by a positive direct antiglobulin test using a broad-spectrum antiglobulin reagent. More precise characterization of the coating immunoprotein is achieved with antiglobulin sera specific for immunoglobulin heavy chains and complement components. With these monospecific reagents, the red cells of 56% of patients show coating with IgG and complement (C3), and 35% with IgG alone. Nine percent are coated with complement alone, and IgA or IgM coating is demonstrated on rare occasions. In SLE, the red cell coating is almost always IgG and complement, and in methyldopa-induced hemolytic anemia, IgG alone.

Very small amounts of IgG can nearly always be demonstrated by sensitive techniques on the surface of red cells in which specific antiglobulin testing reveals coating by complement alone. Small amounts of IgG have also been demonstrated by similar methods on red cells from patients with 'Coombs' negative' warm antibody AIHA, in which the cells do not agglutinate with the usual broad spectrum and specific antiglobulin sera. The amount of IgG on the red cell surface, as measured by the strength of agglutination in the conventional direct antiglobulin test, correlates poorly with the rate of red cell destruction in many cases. More sensitive techniques for measuring cell-bound IgG provides better correlation. Results of sequential quantitative antiglobulin tests on the red cells of individual patients, however, can often be related to fluctuations in severity of the hemolytic process.

It should be remembered that there are a number of causes of a positive direct antiglobulin test besides AIHA. Drugs are of particular importance, methyldopa being the most common cause of a positive direct antiglobulin test in the absence of hemolysis. Weakly positive tests due to complement coating are occasionally seen in ill-patients, possibly as a result of immune complex absorption on the red cell surface with complement fixation.

Antibodies in the Serum

The serum antibodies are usually incomplete antibodies, demonstrated by the indirect antiglobulin test or by the use of enzyme-treated red cells. They coat red cells optimally at 37°C but do not directly agglutinate, except in rare cases. Using the indirect antiglobulin test, serum antibody is detected in 60–80% of cases. With more sensitive enzyme techniques, positive results are obtained in almost all affected patients. If serum antibodies cannot be demonstrated, a drug etiology for the AIHA should be suspected. The antibodies are usually IgG, and frequently have blood group specificity within the Rh system. Thirty percent of red cell eluates from affected subjects show Rh specificity if a panel of commonly occurring red cell types is used. If Rh null red cells are used, a further 35% show some Rh specificity against a 'core' Rh antigen. The most commonly encountered Rh specificity is anti-e. Rh specificity is more frequently shown in cases with IgG alone than with IgG plus complement red cell coating. Antibodies directed against other blood group antigens, e.g. U, LW, Kpb, Wrb, occur occasionally.

Studies on the subclass specificity of eluated IgG antibodies have shown that the majority are IgG1. Most antibody molecules are polyclonal with mixed κ and λ light chains.

Red Cell Destruction—Site and Mechanism

Red cell destruction in warm antibody AIHA is mainly extravascular by the macrophages of the spleen and to a much lesser extent the liver. Destruction within the circulation (intravascular hemolysis) may occur when the hemolytic process is particularly acute, and it is likely that even in chronic warm antibody AIHA there is always a minor element of intravascular red cell destruction. The exact mechanism of red cell destruction is not known with certainty, but the following sequence of events seems most likely.

Extravascular Destruction (Fig. 8.11)

Red cells coated with IgG antibody are preferentially destroyed in the spleen. They attach to macrophages which have surface receptors for the Fc fragments of IgG subclasses IgG1 and IgG3, and are either completely phagocytosed or lose a small part of their membrane to the phagocytosed or lose a small part of their membrane to the phagocytic cell and are rendered spherocytic. The spherocytic cells are released from the macrophages and destroyed prematurely on subsequent recirculation through the spleen.

Red cells coated with complement at the C3b stage may also be phagocytosed by macrophages following adherence to a C3 receptor site on the macrophage surface. Only a small number of red cells are destroyed through this relatively ineffective mechanism. Most are released from the macrophages and resume circulation when the C3b is cleaved by factor I (C3b inactivator), leaving C3d on the red cell surface. Phagocytosis by this mechanism takes place throughout the reticuloendothelial system (especially in the liver) and not preferentially in the spleen. The relationship between the amount of complement on the red cell surface and the degree of hemolysis is highly variable. Little or no hemolysis may occur in spite of the presence of large amounts of bound complement.

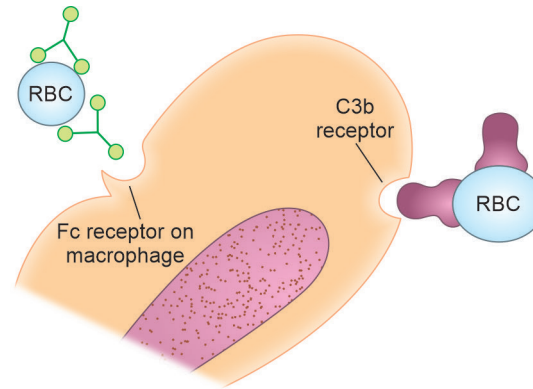


Fig. 8.11: Schematic view of interactions between antibody or complement-coated red cells and specific receptors on the macrophage surface. Red cells coated with IgG1 or IgG3 attach via macrophage Fc receptors, and those coated with C3b via complement receptors. Most red cells attached by the latter mechanism are released following cleavage of surface C3b to C3d (for which there are no macrophage receptor sites) factor I (C3b inactivator)

Intravascular Destruction

The rare finding of hemoglobinuria and hemosiderinuria in warm antibody AIHA indicates the presence of intravascular hemolysis. In these cases, complement fixed on the red cell surface by the IgG or IgM antibody is activated through the complete sequence to C9, which brings about cell lysis. Why the activation of the complement sequence terminates at the stage of C3 in most cases, and does not go on to completion, is not fully understood.

Diagnosis

Diagnosis is based on the demonstration of the typical findings of a hemolytic anemia—jaundice, splenomegaly, anemia, reticulocytosis, and a raised serum bilirubin—with a positive direct antiglobulin test. However, it must be realized that jaundice is absent in 25% of patients.

Once the diagnosis is established, a search must be instituted for a cause. Bone marrow aspiration and trephine biopsy are indicated in most cases, and a careful search for evidence of leukemia, malignant lymphoma, and megaloblastic red cell change should be made. Tests for the presence of SLE are important, and in acute cases in children and young adults, evidence of viral infection should be sought by appropriate cultures and serological studies.

Course and Prognosis

The course of the idiopathic form of disease is exceedingly variable and thus forecasting the outlook in a particular patient is difficult. Most patients respond, at least initially, to one or other form of treatment. In chronic cases, active hemolysis may continue for years, the degree of disability being proportionate to the anemia. Spontaneous remissions occasionally occur; for this reason, the results of treatment are not always easy to assess. No patient can be said to be permanently cured if the antiglobulin test remains positive, even though the hemoglobin, reticulocyte count, and serum bilirubin values are normal. The possibility of relapse always remains, especially following infection.

In secondary cases, the prognosis depends largely on the underlying disease, which in many cases is itself eventually fatal.

Therapeutics

Acute cases in children with moderate anemia may recover without any treatment except for bed-rest. In children with more severe disease, and in most adults corticosteroids, blood transfusion and splenectomy form the main basis of treatment. Immunosuppressive drugs may be used in certain cases.

Corticosteroids induce a prompt reduction in the rate of hemolysis in about 80% of patients. Thus therapy with prednisone, either alone or combined with blood transfusion in more severe cases, is the initial treatment of choice. Response to corticosteroids cannot be correlated with any particular pattern of red cell immunoglobulin coating as shown by specific antiglobulin testing. There is considerable individual variation in the dosage required to induce and maintain remission. The initial dose of prednisone should be large, e.g. 60–80 mg daily. Parenteral hydrocortisone may be necessary in acutely ill-patients. In general, the hemoglobin stabilizes within a week and then slowly rises to near normal or normal levels. The reticulocyte count falls as improvement occurs, but the fall is sometimes preceded by an initial transient rise. Treatment should be continued for at least three weeks before being considered ineffective. When remission occurs, dosage should be gradually reduced to the minimum necessary to maintain a hemoglobin level of a least 11 g/dL. The direct antiglobulin test usually remains positive for many months, although its titer may diminish. Serum antibody titres slowly fall over a period of 3–6 months. Some patients require no maintenance therapy; others require 5–15 mg prednisone, or more, daily. Second daily corticosteroid dosage may reduce the frequency of drug side-effects. In patients requiring large maintenance doses (in excess of 15 mg) over a long period of time splenectomy should be considered. When relapse occurs after remission, higher doses are reinstituted. Corticosteroids appear to reduce the degree of hemolysis in AIHA by

decreasing antibody production and by inhibiting the clearance of antibody-coated red cells by the macrophages of the reticulo-endothelial system.

Blood transfusion is not required in patients with only moderate anemia, but concentrated red cells should be used in severe cases whenever necessary to maintain a hemoglobin level and blood volume compatible with life. Transfusions should be kept to a minimum, especially when they appear to be ineffective. The transfused cells are often rapidly destroyed and the hemoglobin rise is transient, usually lasting only a matter of hours.

The autoantibody on the patient's red cells and/or in the serum may cause difficulty both in blood grouping and cross-matching, and the tests should be performed by an experienced blood transfusion serologist. It is essential as a first step to exclude the presence of alloantibodies in the serum, and an attempt should then be made to establish the specificity of the autoantibody. In some cases, limited specificity within the Rh system may be demonstrated, and it is possible to transfuse units negative for the relevant antigen. More frequently, the auto-antibodies are apparently nonspecific, and all units of the correct ABO and Rh group cross-matched by the indirect antiglobulin and enzyme techniques show some incompatibility. When this occurs, the selection and slow administration of donor red cells showing the least incompatibility is usually advised. The patient's plasma hemoglobin should be monitored during the transfusion by performing a microhematocrit at regular intervals and inspecting the plasma color in the centrifuge tube.

Splenectomy results in complete or near complete remission in about 60% of patients. Splenectomy should be reserved for: (a) idiopathic cases which have not responded to adequate treatment with corticosteriods, and for secondary cases which have not responded to treatment of the underlying causative disorder; and (b) patients who have responded to corticosteroids, but in whom after some months there are still signs of activity, and who require large maintenance doses to sustain a reasonable hemoglobin level; even if a complete remission does not follow splenectomy, it is sometimes possible to control the anemia with smaller corticosteroid doses than were required before splenectomy.

It is not possible to predict definitely from the clinical or hematological findings whether a particular patient will respond to splenectomy. Patients who respond well to corticosteroids are more likely to respond to splenectomy than those who do not. *In vivo* studies with ^{51}Cr-labelled red cells using surface counting techniques may be helpful. A good response to splenectomy can be anticipated in patients with excess counts over the spleen; however, even in patients in whom significant destruction is occurring in the liver, splenectomy may be beneficial in that it may be possible to maintain a symptomatically comfortable hemoglobin level with lower doses of steroid. The direct antiglobulin test often remains positive following splenectomy, irrespective of the clinical results. However, the serum antibody titer may fall when remission occurs. The patient who has a complete remission following splenectomy is not necessarily permanently cured as relapse may occur weeks, months, or even years later.

At operation the abdomen should be explored for enlarged lymph nodes and ovarian dermoid cysts. Accessory spleens should be searched for and removed. Histological examination of the spleen may give the first evidence of an underlying disorder, e.g. malignant lymphoma. Post-splenectomy thrombocytosis is frequently seen, particularly in patients who remain anemic, and heparin therapy may be necessary when the platelet count rises to very high levels.

Immunosuppressive therapy with oral azathioprine in a dose of 2–2.25 mg/kg/day, or with cyclophosphamide, 1.5–2.0 mg/kg/day, has been used with some success. Careful monitoring of the neutrophil count is necessary. The occasional development of leukemia and malignant lymphoma in patients receiving long-term azathioprine suggests that the drug should not be used unless absolutely necessary. It is generally reserved for those patients who fail to respond adequately to splenectomy and in whom further corticosteriod administration is without effect or is required in high dosage. Oral folic acid, 5 mg daily, should be given to all patients with continuing hemolysis.

Treatment of the causative disorder in secondary AIHA. In most secondary cases, the initial management is the same as for idiopathic cases, and the causative disorder is treated either concurrently or subsequently. The response to treatment varies; many patients respond despite persistence of the causative disease, although some do not. In patients with mild anemia, treatment of the causative disease may be tried first; remission of the anemia occasionally follows relief of the causative disorder. If AIHA develops in a patient with a previous history of malignant lymphoma or other neoplasm, a thorough check for recurrent disease should be made.

RECAPITULATION

- Acquired disorder, common in adults
- *Clinical* Onset is insidious, evidences of intravascular hemolysis present.

Common Features

- Weakness
- Splenomegaly
- Hepatomegaly
- Lymphadenopathy
- Dizziness
- Fever
- Jaundice

Rarely may have

- Dyspnea
- Cough
- Weight loss
- GI complaints
- Anorexia
- Dark urine
- Angina
- Confusion
- Thyromegaly
- Edema
- Cardiac failure
- Pallor

Blood Picture

- Hemoglobin and hematocrit—variable, depending upon severity of anemia
- MCV often in macrocytic range (reflects younger population of cells)
- Reticulocytosis or reticulocytopenia
- Normoblastemia
- Autoagglutination (heavy coating by immunoglobulins)
- TLC variable, usually monocytosis in peripheral smear with or without erythrophagocytosis
- DLC, shift to the left in acute cases
- Platelet count usually normal but reduced in Evan's syndrome

- Serum bilirubin raised, 2.5–5 g% predominantly indirect reacting bilirubin.
- In acute fulminant cases typical characteristics of intravascular hemolysis occur
- Slight increase in osmotic fragility
- Spherocytes seen in peripheral smear
- Red cell serum folate diminished
- Red cell destruction occurs extravascularly, done by splenic macrophages mainly.

Immunology

- Autoantibodies demonstrated *in vitro* in most cases
- Antibody is found on red cell surface and in serum (surface antibodies revealed by direct Coombs' test)
- IgA immunoglobulin deficiency.

Treatment

- Steroids
- Transfusion
- Splenectomy
- Immunosuppression
- Treatment of causative disorder (stop the offending agent)
- Patient is said to be cured when positive Coombs' (direct) test becomes negative.

COLD ANTIBODY AIHA

Cold Antibody Autoimmune Acquired Hemolytic Anemia

This is autoimmune acquired hemolytic anemia due to cold antibodies, i.e. autoantibodies that react best with red cells at temperatures below 37°C. It is less frequent than the warm antibody type. Two forms are recognized.

Cold hemagglutinin disease (CHAD) is characterized by a hemolytic anemia of varying severity due to autoantibodies that act as red cell agglutinins at low temperatures. Paroxysmal cold hemoglobinuria (PCH) is characterized by episodes of acute hemolysis due to auto-antibodies that act primarily as red cell lysins at low temperatures. Both CHAD and PCH may be idiopathic or secondary to an underlying illness.

Has two forms:
- Cold hemagglutinin disease (CHAD)
- Paroxysmal cold hemoglobinuria (PCH)

Cold Hemagglutinin Disease (CHAD)

Idiopathic type of CHAD occurs in adults aged over 50 years, and is rare in children. Both sexes are equally affected, and most patients run a chronic course.

The secondary form occurs as a rare complication of:
- malignant lymphomas, chronic lymphocytic leukemia,
- Waldenstrom's macroglobulinaemia or SLE, or as an acute transient hemolytic anemia secondary to infectious mononucleosis or *Mycoplasma pneumoniae* infection.

Clinical Features

In idiopathic CHAD and secondary CHAD associated with the malignant lymphomas, chronic lymphocytic leukemia, or SLE, the onset is insidious with symptoms of anemia. Two clinical patterns are recognized, depending on the thermal range of the antibody.

Some patients experience episodes of acute intravascular hemolysis and hemoglobinuria in cold weather but maintain a normal hemoglobin level when the weather is warmer.

Other patients have a well-compensated chronic hemolytic anemia with a mild-to-moderate reduction in hemoglobin, perhaps slightly worse in the cold weather, but only rarely experience attacks of acute hemolysis. Agglutination of the patient's red cells as they traverse the cooler peripheral areas of the circulation results in varying degrees of obstruction of the microcirculation and symptoms and signs of cold sensitivity, e.g. Raynaud's phenomenon, acrocyanosis and, rarely, peripheral gangrene. These phenomena are more likely to occur in cold weather and are often absent in temperate climates.

Very occasional patients with idiopathic CHAD develop a malignant lymphoma as a terminal complication. More frequently, CHAD develops in a patient with a preexisting lymphoma .

Post-infectious CHAD, as seen in infectious mononucleosis and a typical pneumonia caused by *Mycoplasma pneumoniae*, typically has an acute onset in the second or third week of the infective illness. There is evidence of acute intravascular hemolysis with jaundice and splenomegaly. Cold sensitivity is usually not seen, and the hemolysis resolves in 2–3 weeks in most cases.

Blood Picture (Fig. 8.12)

The usual blood picture is of an anemia with red cell agglutination, reticulocytosis, and a positive direct antiglobulin test.

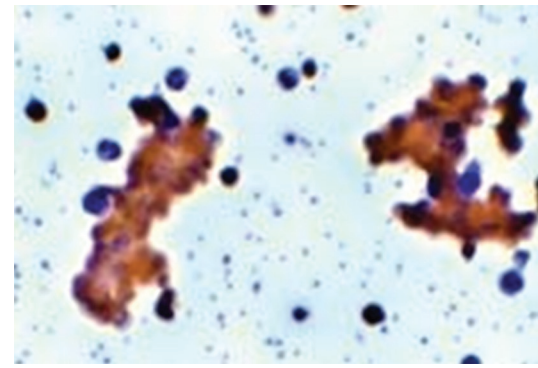


Fig. 8.12: Cold antibody AIHA, peripheral smear

The red cell agglutination is the outstanding diagnostic feature. It is seen on blood films prepared from capillary specimens or from blood collected into an anticoagulant, and is often visible macroscopically in the collection tube. The agglutination may be avoided if the blood specimen is kept at 37°C before spreading and the slides are prewarmed. The hemoglobin level is reduced, but it rarely falls below 8 g/dL, and may be normal. It may fall to low values in acute exacerbations. Spherocytosis is usually present, but is less marked than in warm antibody AIHA. The reticulocyte count is mildly increased, and polychromatic macrocytes and occasional nucleated red cells are seen on the blood film. The white cell count and platelet count are usually normal. The serum bilirubin is mildly elevated and the plasma hemoglobin increased in active disease. Hemosiderin is often found in the urine in both active and inactive phases. The red cell agglutination interferes with the function of automated cell counters, and spuriously high MCV results are obtained. Serum and red cell folate levels may be reduced.

Immunology

Most cases of CHAD are caused by an autoantibody referred to as anti-I. This antibody reacts with the I antigen which is found on the surface of nearly all adult human red cells but not on the surface of fetal red cells. The antibody is a complete antibody which agglutinates red cells with increasing strength as the temperature is lowered to 4°C, but is inactive at 37°C. Its lytic activity is much less marked than that of the Donath–Landsteiner

antibody of paroxysmal cold hemoglobinuria. Most patients with idiopathic CHAD or CHAD secondary to leukemia, malignant lymphoma, or SLE have very high cold agglutinin titres, e.g. from 64000 to 512000. The agglutinin titres in CHAD secondary to *Mycoplasma pneumoniae* infection are usually not as high as in idiopathic CHAD. The agglutinating antibody in CHAD associated with infectious mononucleosis and some cases of malignant lymphoma is anti-I. This antibody strongly agglutinates umbilical cord red cells at low temperatures but is relatively inactive against adult red cells. The anti-I antibodies of idiopathic CHAD and most types of secondary CHAD are IgM in type with light chains. An M-band is often evident on serum electrophoresis, and immunoelectrophoresis confirms that the abnormal band is a monoclonal IgM protein. The serum IgM level is elevated to about 6 g/l, but IgG and IgA are usually normal. The anti-I antibody of CHAD secondary to Mycoplasma infection is also IgM, but is usually polyclonal with mixed and light chains.

The direct antiglobulin test is positive if the blood specimen is drawn and kept at 37°C, and washed in warm saline before testing. The positive test is due to the presence of complement (C3d) on the red cell surface as demonstrated by specific antiglobulin sera. Serum complement is often depleted.

Site and Mechanism of Red Cell Destruction

As in warm antibody AIHA, red cell destruction in CHAD occurs both in the circulation and in the reticuloendothelial system. The IgM antibody fixes complement to the patient's red cells in the cooler peripheral areas of the circulation. When the red cells return to areas of the body where higher temperatures prevail, the anitbody dissociates from the red cell surface, leaving the complement still bound. Probably depending on the amount of complement and other factors, the complement sequence either terminates at the C3b stage or, less commonly, goes on to completion with intravascular lysis. The C3b-coated cells adhere to hepatic macro-phages, and their subsequent fate is similar to that of the complement-coated cells in the warm antibody hemolytic anemias.

Prognosis and Treatment

Idiopathic CHAD usually progresses slowly, and many patients remain relatively well, particularly if the upper thermal range of the antibody does not exceed 28°C. Protection from the cold weather, or a move to a more temperature climate, may be all that is needed. Corticosteroids and splenectomy are rarely of any benefit. Chlorambucil is of value in some patients, and usually leads to a reduction in the level of IgM in the serum. Occasional patients progress rapidly and require blood transfusion. Concentrated red cells are preferable, and the patient should be kept warm and the donor units warmed to body temperature before and during administration. Plasmapheresis has been used with success in some very severe cases. CHAD associated with infective illnesses is usually self-limiting.

RECAPITULATION

Characterized by hemolytic anemia of varying severity due to autoantibodies which act as red cell agglutinins at low temperatures (<32°C).

Etiology

CHAD
- Primary, equal sex incidence, has a chronic course
- Secondary

Diseases in which cold agglutinins may occur.

Polyclonal Cold Agglutinins

- *Mycoplasma pneumoniae* infections
- Infectious mononucleosus
- Cytomegalovirus infection
- Listeriosis
- Mumps
- Subacute bacterial endocarditis
- Syphilis
- Trypanosomiasis
- Malaria
- Tropical eosinophilia
- Collagen vascular and immune complex diseases
- Angioimmunoblastic lymphadenopathy

Monoclonal Cold Agglutinins

- Chronic cold agglutinin disease
- Waldenstrom's macroglobulinemia
- Lymphomas
- Chronic lymphocytic leukemia
- Kaposi's sarcoma
- Myeloma
- *Mycoplasma pneumoniae* infections (rare)

Characteristics of Cold Agglutinins

- Almost all cold agglutinins are IgM
- On combining with red cells the cold agglutinins fix complement and can be shown to lyse red cells in ultra if the pH is adjusted to 6.5–7.0 (almost all cold agglutinins bind complement).

Two Clinical Patterns are Seen

- Hemolysis at low temperatures but not at 37°C
- Chronic compensated hemolytic anemia with episodes of acute attacks.

Clinical Features

- This is a disease of the elderly
- Acrocyanosis: Is caused by intracapillary agglutination of red cells in areas of body that are cooled to temperatures encompassed by the thermal range of the antibody in question. This leads to skin discoloration which may vary from a sick-looking white to deep blue-violet. The color changes may be accompanied by numbness and occasionally by pain.
- Gangrene may occur secondary to vascular obstruction (coupling of cold agglutinins and cryoglobulins)
- Ice cube test for acrocynosis: Placing an ice cube over the palm will produce a circumscribed area of intravascular agglutination and acrocyanosis in the hyperemic palm.
- Ehrlich finger test: occludes the venous return of a finger with a rubber band, immerse the finger in cold water (20°C) for 15 minutes: at the same time immerse another finger in 37°C water (control). The centrifuged capillary blood obtained from cold finger would be markedly hemolyzed.

Blood Picture

- Anemia, hemoglobin and hematocrit depend on the severity of disease
- Red cell agglutination of peripheral smear (avoidable by raising temperature to 37°C)
- Reticulocytosis
- Regenerative macrocytosis
- Hyperbilirubinemia (rarely more than 3 mg percent, mainly indirect)
- Positive (direct) Coombs' test (because of presence of C3d on red cell surface as shown by specific antiglobulin sera).
- Complement levels decreased during episode.

Paroxysmal Cold Hemoglobinuria (PCH)

This (PCH) is a rare disorder characterized by attacks of hemolysis with hemoglobinuria on exposure to cold, either local or general. It results from the development of a cold autoantibody with strong lytic activity.

The disorder may be idiopathic or secondary to viral illnesses such as mumps, measles, or influenza. In children with viral illnesses, exposure to cold is not necessarily involved in precipitation of the hemolysis. In adults, the illness is usually chronic and takes the form of episodic hemolysis and hemoglobinuria precipitated by exposure to cold.

The degree of chilling required to cause hemoglobinuria varies; in some cases it is only slight, and may be limited to one part of the body, e.g. immersion of hands in cold water or taking a cold drink. After a period of minutes to several hours, the patient develops pain in the back and legs, sometimes with abdominal cramp and headache, and then a rigor with a sharp rise of temperature. The anemia is usually severe (although short-lived), and red cell spherocytosis, erythrophagocytosis, and leukopenia are present on the blood film. The first specimen of urine passed after the rigor usually contains hemoglobin; hemoglobinuria disappears in several hours. The spleen may become palpable at the time of attack, and transient jaundice is common on the day following the attack. Rarely, the degree of hemoglobinemia is not sufficient to cause hemoglobinuria, and only constitutional symptoms occur.

The characteristic finding in the blood is a bithermic cold hemolysin which can be demonstrated by the Donath–Landsteiner test. The principle of the test is that the hemolysin in the patient's blood attaches to the red cells when the blood is chilled, and the sensitized cells are then hemolysed by complement when the blood is warmed to 37°C. The cold hemolysin is a complement-binding IgG antibody, and is usually found to have anti-P blood group specificity. The direct antiglobulin test is positive only during the episode of hemolysis. The positive result is due to the presence of complement on the red cell surface.

Attacks of acute hemolytic anemia on exposure to cold with sudden passage of hemoglobin in urine.

Types

- Primary
  - Idiopathic
- Secondary
  - Viral infections
  - Measles
  - Mumps
  - Influenza
  - Bacterial infection
  - Syphilis

Etiology and Pathogenesis

The D–L antibody (Donath–Landsteiner) is a strong hemolysin even in low concentrations. It is said to be 'biphasic', as chilling to 0°C, followed by warming to body temperature (D–L test) leads to most efficient lysis. The complement system is involved in the lysis. Since the D-L antibody is an IgG immunoglobin, red cells sensitized with it will give a positive reaction in Coombs' test if it is carried out in cold. The antibody was classically described earlier in patients of tertiary syphilis (a number of other conditions have since been identified). Genetic factors are also said to play a part in PCH.

Clinical

- Most striking manifestation is the passage of darkbrown or black urine after local or generalised exposure to cold.
- Systemic manifestations appear any time from a few minutes to 8 hours after chilling and may consist of:
  - Aching and pain in the back, legs, and abdomen
  - Cramps
  - General malaise
  - Headache
  - Vomiting
  - Diarrhea and
  - Severe shaking chills and fever.
- Following the attack the patient is weak, pale, and may be slightly icteric.
- Hepatosplenomegaly may sometimes occur.
- Vasomotor disturbances such as urticarial rashes, acrocyanosis, etc. have been described.
- Recovery from attack is usually rapid and between the attacks the patient may be entirely symptom free.

Diagnosis

Blood

- Features of acute intravascular hemolysis are seen
- During a sudden massive attack
  - Hemoglobin may drop rapidly
  - Plasma becomes markedly red
- Hemoglobinuria occurs once the haptoglobin is saturated, at this time—methemalbumin is also detectable in the plasma with a mild to moderate elevation of indirect bilirubin
- No diagnostic morphological changes occur in red cells, but erythrophagocytosis is a common feature in acute attacks (Fig. 8.13).
- Leukopenia occurs early, later there may be myeloid leukemoid reaction
- Continuous cold weather exposure may lead to chronic hemolytic anemia with hemosiderinuria

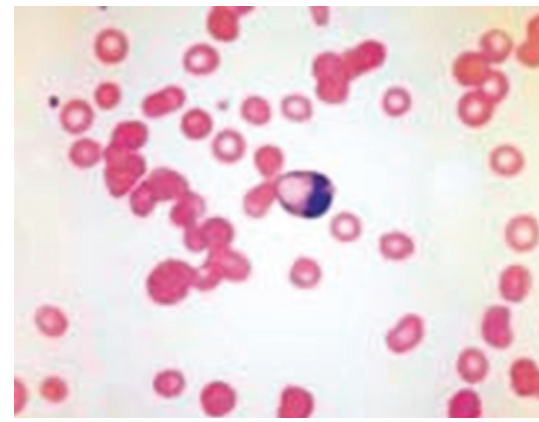


Fig. 8.13: PCH, smear shows erythrophagocytosis

Urine

- The urine contains hemoglobin and methemoglobin giving it a dark-red, brown, or almost black appearance.
- There may be red cell casts and albuminuria occasionally.
- Urinary abnormality may be confined to the first two or three specimens obtained after an attack.

Serologic Findings

- Positive Coombs' test during the attack
- Positive D–L antibody test: Chill the blood, take it back to 37°C. hemolysis takes place.

Differentiation from CHAD is given in Table 8.6.

Differences between warm and cold AIHA are given in Table 8.7.

Table 8.6: Comparison of PCH and CHAD		
	PCH	*CHAD*
Patient population	Children or young adults	Elderly or middle-aged
Pathogenesis	Following viral infection usually	Idiopathic/lymphoproliferative disorder/following *Mycoplasma pneumoniae* infection
Clinical features	Hemoglobinuria: Acute attacks upon exposure to cold (symptoms resolve in hours or days)	Acrocyanosis/autoagglutination of blood at room temperature
Severity of hemolysis	Acute and rapid	Chronic and rarely severe
Hemolysis	Intravascular	Extravascular/intravascular
Autoantibody	IgG (anti-P (specificity) (biphasic)	IgM (anti 1/1) (monophasic)
Direct antiglobulin test	3+ (polyspecific Coombs'sera)/negative IgG/3–4 + C3 monospecific Coombs' sera	3+ (polyspecific Coombs' sera)/negative lgG/3–4+ C3 monospecific Coombs' sera
Thermal range	Moderate (<20°C)	High (up to 30–31°C)
Titer (4°C)	Moderate (<64)	High (>1000)
Donath Landsteiner	Positive	Negative
Treatment	Supportive (disorder terminates when underlying illness resolves)	Avoid the cold

Table 8.7: Differences between warm AIHA and cold AIHA			
	Warm AIHA	*Cold AIHA*	
		CHAD	*PCH*
lg class	*IgG*	*IgM*	*IgG*
Immunochemical characteristics	Polyclonal	Monoclonal	Polyclonal
Optimal reaction temperature	37°C	0–4°C	0–4°C
Antibody specificity *in vivo* — hemolysis	anti-Rh rare	anti-I or P minor	anti-P major
Serological behavior *in vitro*	Incomplete	Agglutinin	Biphasic hemolysin
Protein on red cell surface	IgG 35% IgG + C 56% C 9%	C–100%	C–100%

Paroxysmal Nocturnal Hemoglobinuria (PNH) (*Marchiafava-Micheli Syndrome*)

PNH is classically characterized by attacks of intravascular hemolysis and hemoglobinuria, which occur chiefly at night. However, such a classic pattern may not be seen in all cases. Hemolysis in PNH because of an obscure structural defect of the red cell membrane that makes the cells unusually susceptible to the lytic action of complement.

Etiology and Pathogenesis

As established by cross-transfusion studies, in PNH there is an intrinsic abnormality of the red cells, as normal red cells survive normally in patients with PNH while PNH red cells have shortened life span within the patient or a normal recipient.

Three distinct red cell populations are seen. Those with:

a. Extreme sensitivity (PNH-III)
b. Intermediate sensitivity (PNH-II)
c. Complete insensitivity (PNH-I)

The proportion of these cells varies from patient to patient. Excessive lysis in acidified human serum is a characteristic feature of PNH cells. The optimal pH for this reaction is 6.8–7.1 with a marked reduction in the degree of hemolysis below or above this range. The optimal temperature for acid hemolysis is between 37°C and 40°C.

It is likely that the increased sensitivity of PNH cells to complement is related to a structural or biochemical defect of the cell membrane, but the nature of defect is still uncertain. Certain changes as revealed by electron microscopy include a patchy pitted red cell surface and many cleft-like faults. In addition, electrophoresis of red cell membrane proteins has also been found to be abnormal. The most consistently demonstrated abnormality in membrane protein is a decrease in membrane acetylcholinesterase (AChE) activity.

PNH granulocytes (polymorphs) and platelets appear to share the membrane defect of PNH red cells, since they are also much more sensitive to lysis by complement or antibodies than are normal cells.

PNH is now grouped under 'myelodysplastic' diseases and it has a definite relationship with aplastic anemia and may rarely progress on to leukemia.

Clinical

- PNH begins insidiously, has a prolonged and a constant course, usually detected in third to fifth decades. Both sexes are affected equally and has no racial predilection.
- Diagnosis is often delayed and usually 2½–3 years elapse between onset of symptoms and diagnosis establishment.
- Hemoglobinuria—nocturnal hemoglobinuria when present occurs as the result of an increase in hemolysis during sleep. It is not due to night time per se, since the rhythm can be reversed if the patient stays awake at night and sleeps by day. In patients suffering from nocturnal hemoglobinuria the urine is usually discolored in the morning and clears during the day.
- Episodic hemolysis—In addition to sleep-related rhythmicity in hemolysis and hemoglobinuria, most patients suffer from irregularly recurring exacerbations in hemolysis. Exacerbations may be precipitated by:
  - Infections
  - Operations
  - Transfusions
  - Iron salts intak or
  - Vaccinations

  (Hemoglobinuria is not related to cold exposure)

  The milder attacks may pass unnoticed but severer ones are characterized by:
  - Substernal, lumbar or abdominal pain
  - Drowsiness, general malaise
  - Fever and headaches

  The abdominal pain may be colicky and may last for one or two days. The abdomen may be tender to examination, especially in the increased symptoms on rebound. The back pain resembles that found in patients with other types of intravascular hemolysis. It is maximal in lumbar region. Headaches may last for days.

Complications

The most serious complications include marrow aplasia, thromboses, and infections.

The association between aplastic anemia and PNH has been clearly documented.

PNH is associated with a striking predisposition to intravascular thromboses, especially within the venous circulation. These account for nearly 50% of all deaths in patients with PNH. Fatal thromboses usually involve the portal system or the pain. Abdominal pain—its cause is mysterious. The possibility of an intra-abdominal thrombosis must always be considered. Thrombosis may occur within the portal or mesenteric veins, but progressive diffuse hepatic venous thrombosis (HVT) appears to be particularly common and usually runs a fatal course.

Severe and refractory headaches may be due to small vessel thromboses or they may be premonitory signs of progressive cerebrovascular thromboses. (The release of thromboplastic material from hemolyzed red cells may be responsible for the increased tendency to thrombosis that characterizes PNH).

Painful swallowing occurs in many patients during hemolytic episodes. Infections occur frequently and may by partially attributable to leukopenia or, perhaps to functional defects of leukocytes. Occasionally, PNH has terminated in acute myeloblastic leukemia and has been reported to occur in association with myelofibrosis or erythroleukemia.

Blood Picture (Fig. 8.14)

- Anemia—often less than 6 g% macrocytosis, if hemosiderinuria is there, i.e. iron loss, the red cells can be microcytic hypochromic, also polychromasia is observed
- Reticulocytosis, moderate leukopenia, mild thrombocytopenia is also seen
- HbF occasionally raised
- Hyperbilirubinemia
- Plasma may be golden brown, reflecting presence of increased levels of indirect bilirubin, hemoglobin and methemalbumin.
- Predictably, serum haptoglobins are very low and LDH may be very high during periods of active hemolysis.
- Neutrophil alkaline phosphatase score is low, but normal in aplastic cases
- Hypercoagulability
- Direct Coombs' test may be positive.

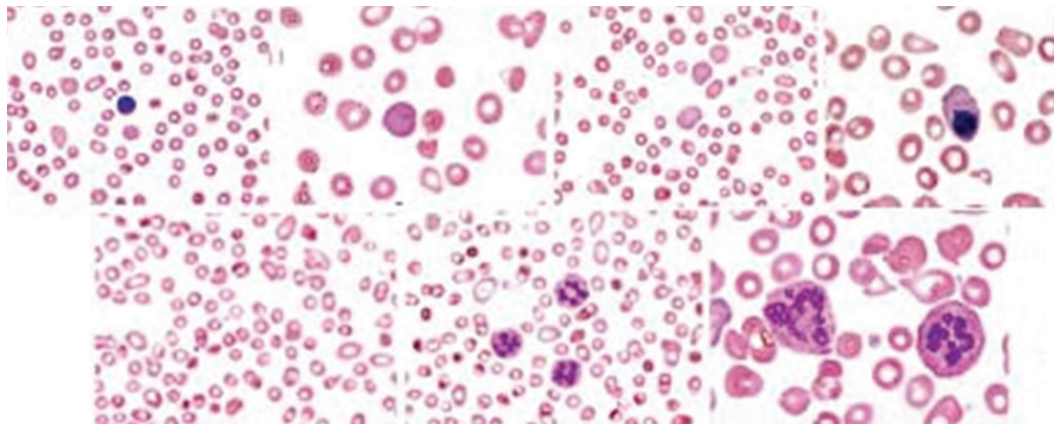


Fig. 8.14: PNH, peripheral smear (see polychromatophilic RBCs)

Bone Marrow

Normoblastic hyperplasia is the typical finding, it is rarely megaloblastic. Mega-karyocytes may be diminished. In pancytopenia, bone marrow is hypoplastic.

Diagnosis

- Hemoglobinuria
- Hemosiderinuria
- Serological tests
  a. Ham's acid serum test—patients red cells will undergo lysis in compatible acidified serum at 37°C (serum may be patient's own), 10–50% lysis implies a positive test.
  Differential diagnosis—hereditary erythroblastic multinuclearity with a positive acid serum test (HEMPAS)—this gives a negative test with patient's own serum.
  b. Sucrose hemolysis test—Isotonic solution of low ionic strength; if causes hemolysis of more than 10% ascertain diagnosis of PNH.

Treatment

No specific treatment.

HEMOLYTIC ANEMIA DUE TO DRUGS AND CHEMICALS

Many drugs and chemicals may cause hemolytic anemia. They can be subdivided into three groups:

- Those that have a direct toxic action on the red cell; the hemolysis is dose-related and occurs in most normal subjects provided that sufficient dose of the drug is given;
- Those that cause hemolysis as a result of a hereditary metabolic abnormality of the red cells; and
- Those that cause hemolysis by immunological mechanisms.

HEMOLYTIC ANEMIAS CAUSED BY DRUGS

Direct Toxic Action of Drugs and Chemicals Metabolic Abnormality in the Red Cell

- Hereditary enzyme deficiencies
- Unstable hemoglobins

Immune-based Mechanisms

- Immune hemolytic anemia
- Autoimmune hemolytic anemia

AGENTS THAT MAY CAUSE HEMOLYTIC ANEMIA (DRUGS AND CHEMICALS)

Drugs that Regularly Cause Hemolytic Anemia in Normal Subjects by Direct Toxic Action

- Acetylphenylhydrazine
- Phenylhydrazine
- Arsine
- Potassium chlorate
- Chloramines
- Resorcinol
- Copper

- Sodium chlorate
- Formaldehyde
- Sulfanilamide
- Naphthalene
- Sulfapyridine
- Para-aminosalicylic acid
- Sulfasalazine
- Phenacetin
- Sulfones
- Phenazopyridine

Drugs that Cause Hemolytic Anemia in Subjects with Hereditary Metabolic Abnormalities of the Red Cell (Mainly G6PD Deficiency)

- Analgesics—Acetanilide
- Sulfones—Dapsone, thiazole sulfone
- Antimalarials—Pamaquin, pentaquine, primaquine
- Nitrofurans—Nitrofurantoin
- Sulfonamides—Sulfacetamide, sulfanilamide, sulfapyridine, sulfasalazine
- Miscellaneous—Acetylphenylhydrazine, methylene blue, nalidixic acid, nitrofurans, naphthalene (mothballs), niridazole, phenylhydrazine, toluidine blue, trinitrotoluene.

Drugs that Cause Hemolytic Anemia by Immune Based Mechanisms

- Immune
- Quinine
- Amidopyrine
- Rifampicin
- Antazoline
- Sulfasalazine
- Cephalosporins
- Stibophen chlorpromazine
- Sulfonamides
- Chlorpropamide
- Teniposide
- Cisplatin
- Tetracycline
- Dipyrone
- Thiazides
- Erythromycin
- Thiopentone
- Insulin
- Tolbutamide
- Isoniazid
- Triamterene
- Nomifensine
- Para-aminosalicylic acid
- Paracetamol
- Penicillin
- Phenacetin
- Quinidine

- Autoimmune
- L-dopa
- Mefenamic acid
- Methyldopa
- Procainamide
- Additionally, many of the agents that regularly cause hemolysis in large doses may cause hemolysis in G6PD-deficient subjects in smaller doses.

HEMOLYTIC ANEMIA DUE TO DIRECT TOXIC ACTION

Hemolysis may result on account of: (a) from drug therapy, (b) occupational poisoning, and (c) household poisoning. Most of the drugs and chemicals or their metabolites are powerful oxidants which injure the red cell membrane and interfere with the cell's normal metabolism. They lead to the formation of methemoglobin and to the denaturation of globin, which precipitates in the red cells as Heinz bodies (Fig. 8.15). Typically, the red cells are contracted, distorted, and often spherocytic, reflecting direct injury to the cell membrane by the drug and damage sustained during circulation through the spleen. Many of the drugs and chemicals that regularly cause hemolysis in large doses may cause hemolysis in patients with G6PD deficiency, or an unstable hemoglobin in small doses.

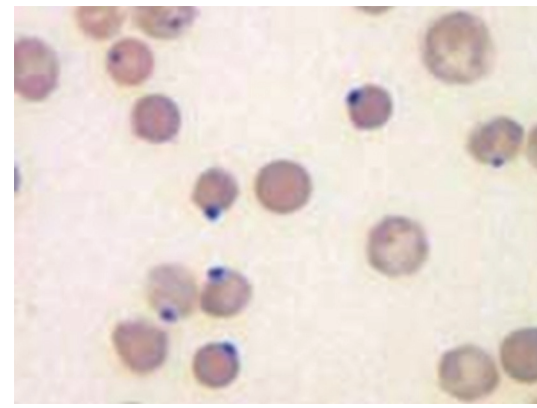


Fig. 8.15: Heinz bodies in peripheral smear

Drug Therapy

Commonly used drugs that have been known to cause this type of hemolysis include the sulphones, which are used in the treatment of leprosy and dermatitis herpetiformis, few sulphonamides, and para-aminosalicylic acid.

Occupational Poisoning

Hemolytic anemia may follow exposure to arsine (arseniuretted hydrogen) in submarines (storage batteries), chemistry laboratories, and factories. Accidental ingestion of phenylhydrazine or acetylphenylhydrazine occasionally occurs in chemistry laboratories, with similar results.

Household Poisoning

Cases of acute hemolytic anemia have been reported in young children who have swallowed mothballs containing naphthalene, and in infants as a result of skin absorption from napkins impregnated with naphthalene; some of these have been associated with G6PD deficiency.

Contamination of Hemodialysis Systems

Several outbreaks of acute hemolytic anemia in patients undergoing chronic maintenance hemodialysis have been caused by contamination of the dialysate by chloramines, which are bactericidal oxidant compounds used for purification of urban water supplies. Contamination of dialysis fluid by the sterilizing agent, formaldehyde, also results in acute hemolysis.

Hematological Picture

The severity of anemia varies and is usually proportional to the dose. It is often accompanied by one or more of the following features which result from the action of the toxic agent on the red cell: (i) irregular contraction and 'blister' or 'bite' cell formation; (ii) spherocytosis; (iii) Heinz bodies; and (iv) methemoglobinemia and sulfhemoglobinemia. Evidence of intravascular hemolysis (hemoglobinemia, hemoglobinuria, and methemalbuminemia) is also usually present.

The presence of the Heinz bodies (Fig. 8.15), methemoglobin, or sulfhemoglobin strongly suggests that the hemolysis is due to a chemical cause. However, their absence does not exclude such a cause, as some substances producing hemolysis do not result in their formation. Neutrophilia, sometimes with toxic granulation, is usual in acute chemical hemolytic anemia, and a moderate platelet increase may also occur. A G6PD screening test and the heat instability test for unstable hemoglobins should be performed. Analysis of urine for drug metabolites may help in cases in which the nature of the poison is uncertain.

DRUG-INDUCED HEMOLYTIC ANEMIA DUE TO HEREDITARY RED CELL ENZYME DEFICIENCIES

Acute hemolytic anemia due to certain drugs administered in usual doses may be associated with inherited red cell metabolic defects, of which by far the most common is a deficiency of glucose-6-phosphate dehydrogenase. With some other enzyme abnormalities, a pre-existing mild hemolytic anemia may be aggravated by drug ingestion.

DRUG-INDUCED HEMOLYTIC ANEMIA DUE TO UNSTABLE HEMOGLOBINS

Exacerbation of hemolysis by drugs has been demonstrated in some patients with hemoglobinopathies due to unstable hemoglobins. Hb-Zurich has been most extensively studied in this respect. The drugs involved have been similar to those causing hemolysis in G6PD-deficient subjects.

IMMUNOLOGICAL DRUG-INDUCED HEMOLYTIC ANEMIA

About 20% of cases of acquired hemolytic anemia due to immune mechanisms are attributable to the administration of drugs. Penicillin and methyldopa are most frequently involved, but a wide range of drugs have the ability to initiate in the susceptible person immunological mechanisms that lead, directly or indirectly, to premature red cell destruction.

Two forms of immunologically medicated drug-induced hemolytic anemia are recognized.

Immune hemolytic anemia in which antibodies are formed against the offending drug or its metabolites. Serum antibodies cannot be demonstrated *in vitro* with normal red cells unless the drug is also present in the test system.

Autoimmune hemolytic anemia in which antibodies are formed against red cell antigens. Serum antibodies can be demonstrated *in vitro* with normal red cells in the presence or absence of the drug from the test system.

A number of cases have been reported in which there is evidence that cell destruction has been caused by both mechanisms acting simultaneously (Table 8.8).

Table 8.8: Hemolytic anemias—immunological drug-induced

Clinical and hematological picture	*Immune (drug adsorption)*	*Immune (immune complex)*	*Auto-immune*
Drug dose	Large	Small	Prolonged dosage
Onset	Rapid to slow	Acute	Slow
Terminates by	Weeks	Days	Week
Hemolysis site	Extravascular	Intravascular	Extravascular
Spherocytosis	Occasional	Usual	Usual
Renal failure	Rare	Frequent	Rare
Previous administration	Often	Often	Not usual
Direct Coombs' test Indirect Coombs' test	Positive	Positive	Positive
Without drug	Negative	Negative	Positive
With drug	Positive, Cell bound	Positive	Positive
Antibody	IgG	IgM, C binding	IgG

DRUG-INDUCED IMMUNE HEMOLYTIC ANEMIA

Two mechanisms appear to be involved in the pathogenesis of this type of drug-induced hemolytic anemia. Some drugs, e.g. penicillin and cephalothin, have a strong affinity for the red cell membrane. When given to the patient in large doses they bind firmly to the membrane protein, and antibodies to the cell-bound drug are formed by the immune system. This is referred to as the drug adsorption mechanism. Other drugs, e.g. quinidine, para-aminosalicylic acid and rifampicin, do not bind firmly to the red cell membrane. Antibodies are formed directly against the drug (probably bound to a serum protein), and adsorption of the immune complex to the red cell surface activates complement, with ensuing cell lysis. This is called the immune complex mechanism.

DRUG ADSORPTION MECHANISM

Penicillin—over 90% of sera from adult subjects contain IgM antibodies which agglutinate normal red cells coated with penicillin *in vitro.* These antibodies are believed to result from the almost universal exposure of the normal population to penicillin in the environment. Penicillin can be detected on the red cell surface of all patients receiving high doses of penicillin, and 3% of these patients develop a positive direct antiglobulin test without evidence of hemolysis. A high concentration of IgG rather than IgM antipenicillin antibody seems necessary for the development of hemolytic anemia, and this occurs in rare patients who receive massive dose of penicillin intravenously over a long period.

The important serological finding in a patient with penicillin-induced hemolytic anemia is a positive direct antiglobulin test due to red cell coating with IgG, in spite of negative tests for the presence of serum antibody. Serum antibody is, however, easily demonstrable if penicillin-coated red cells are used in the *in vitro* test system.

Cephalothin—hemolytic anemia due to cephalothin is rare. The mechanism of hemolysis seems similar to that of penicillin-induced hemolytic anemia, but smaller drug doses are

involved. Cephalothin and other cephalsporins may cause a positive direct antiglobulin test without hemolysis.

IMMUNE COMPLEX MECHANISM

This form of drug-induced hemolytic anemia is rare. The serum antidrug is usually IgM in type, and the immune complex binds complement when adsorbed on the red cell surface. The positive direct antiglobulin test is due to complement, and immunoglobulins are not usually detectable. Serum antibodies are demonstrable only if the offending drug is included in the *in vitro* test system. In some patients, the antibody is directed against a drug metabolite rather than the drug itself, and it is necessary to use the metabolite in the test system to obtain a positive result.

DRUG-INDUCED AUTOIMMUNE HEMOLYTIC ANEMIA

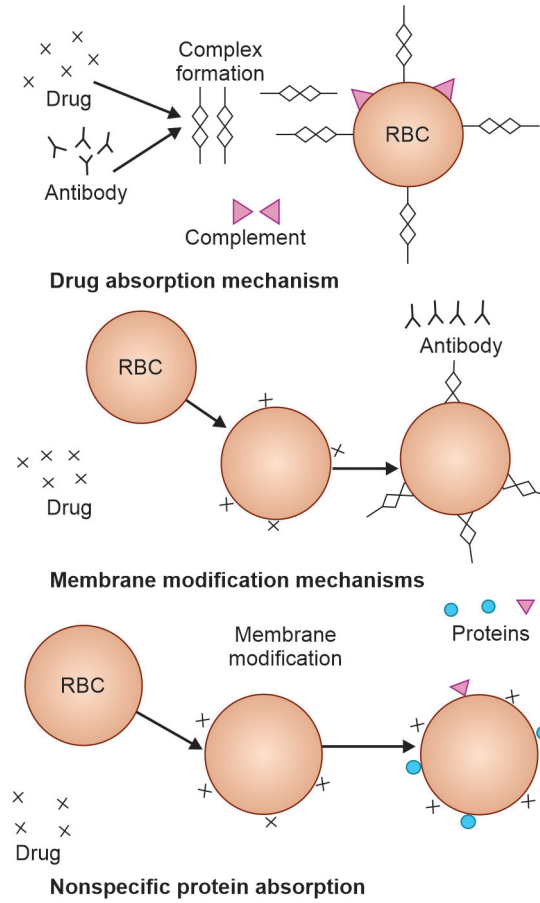
AIHA is a well-recognized complication of treatment with the antihypertensive agent methyldopa. Almost 15% of patients on methyldopa develop a positive direct antiglobulin test without evidence of hemolysis when the drug is given for a sufficient time (commonly from three months to one year) at sufficient dosage. The test gradually becomes negative once the drug is stopped; the time this takes depends on the initial strength of the antiglobulin test, and varies from one month to two years. The finding of a positive direct antiglobulin test in a patient receiving methyldopa is not an indication for cessation of the drug if valid clinical indications for its use are present. Regular clinical and hematological surveillance is desirable in such cases, however, between 0.01 and 0.1% of patients treated with methyldopa develop overt hemolytic anemia. The absence of hemolysis in the majority of patients taking the drug who have a positive direct antiglobulin test may be due to a methyldopa-induced defect in reticuloendothelial function. The anemia generally occurs within 18 months of commencing treatment, but has been diagnosed as early as 4 months and as late as 4 years. Onset of the anemia is usually insidious, and the clinical, hematological, and serological features are similar to those of idiopathic warm antibody AIHA. The direct antiglobulin test is strongly positive due to IgG on the red cell surface and serum antibodies can be demonstrated by the indirect antiglobulin test or by the use of enzyme-treated red cells. Addition of the drug to the test system is not necessary to obtain positive results. Antibody specificity within the Rh system is found in some cases. Following cessation of the drug, the clinical picture improves and the hemoglobin level rises; the hematological picture usually becomes normal fairly rapidly, but the antiglobulin test may take months to become negative.

Corticosteroids are effective and are used if symptoms are troublesome or if a rapid response is required.

About 9% of patients receiving L-dopa develop a positive antiglobulin test without evidence of hemolysis, and cases of hemolytic anemia, similar to those occurring with methyldopa, have been described. Procainamide and the analgesic agent mefenamic acid have been cited as causes of autoimmune hemolytic anemia in a small number of cases, and the drugs rarely give rise to a positive direct antiglobulin test without hemolysis. It should be stressed that the evidence for attributing a drug etiology to a case of warm antibody AIHA is of necessity circumstantial as the autoantibodies involved are identical to those found in idiopathic AIHA (Table 8.9).

MECHANICAL HEMOLYTIC ANEMIA

Damage to red cells may occur by excess physical trauma as they circulate through the vascular system. Such direct injury takes the form of loss of areas of cell membrane, and may be followed by immediate cell lysis. Frequently, however, the injured membrane is resealed with the formation of grossly distorted, though still viable, red cells. The cells are recognized on the blood film as fragmented, contracted, triangular, and helmet-shaped forms, or microspherocytes. The abnormal cells circulate for a short period, but are destroyed prematurely in the circulation or by the reticulo-endothelial cells, and a frank hemolytic anemia ensues. Evidence of both intravascular and extravascular hemolysis is usually present with variable degrees of hemoglobinaemia, hemoglobinuria, methemalbuminemia, hemosiderinuria, and hyperbilirubinemia, depending on the severity of the process. Plasma haptoglobin is reduced or totally depleted, and lactate dehydrogenase is elevated. It should not be forgotten that distorted red cells may be caused by other mechanisms, such as chemical agents and physical agents, e.g. burns.



ETIOLOGY

1. Cardiac hemolytic anemia
2. Microangiopathic hemolytic anemia (due to fibrin deposition in capillaries) (Fig. 8.16)

- Hemolytic uraemic syndrome
- Disseminated intravascular coagulation
- Thrombotic thrombocytopenic purpura
- Disseminated carcinoma
- Malignant hypertension
- Eclampsia
- Hemangiomas

March hemoglobinuria

- Occurs especially in upright position
- Evidences of intravascular hemolysis present
- Possible factors
- Excessive trauma
- Hardness of running surface
- Running distance

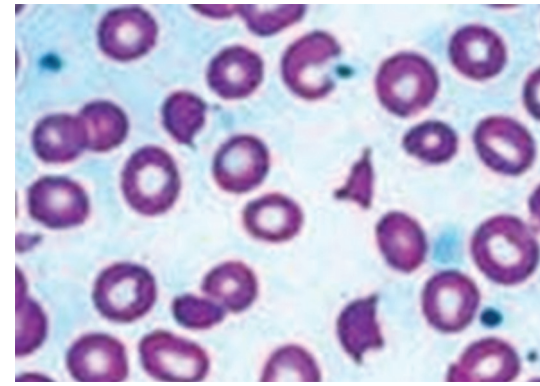


Fig. 8.16: Microangiopathic hemolytic anemia, peripheral smear

- Heaviness of stride
- Protective adequacy of footwear
- Increased temperature in soles.

CARDIAC HEMOLYTIC ANEMIA

Occasionally hemolytic anemia is a complication of open-heart surgical procedures, particularly those involving the use of valve prostheses (mainly aortic but also mitral) and the use of Teflon grafts for the repair of ostium primum and other defects. In cases associted with valve prostheses, malfunction of the prosthesis is almost always present. The hemolysis is considered to be due to direct mechanical trauma to the red cells, consequent on the development of turbulent blood flow in the vicinity of the prosthesis or Teflon graft. In mild cases, hemolysis is compensated and the hemoglobin level is normal. The reticulocyte count and serum bilirubin are slightly elevated; reduction of plasma haptoglobin and an elevated lactate dehydrogenase provide further evidence of subclinical hemolysis, and are useful screening tests.

Marked anemia with evidence of intravascular hemolysis is seen in severe cases. Hemosiderinuria is often a prominent feature. The blood film shows the presence of varying numbers of fragmented cells, similar to those seen in microangiopathic hemolytic anemia. Occasionally, the iron loss from persistent hemosiderinuria results in red cell hypochromia; in such cases the administration of iron may in part relieve the anemia. In patients with mild compensated hemolysis; iron and folic acid supplements and some restriction of physical activity may be enough.

For chronically marked hemolysis, reoperation to correct the functional defect is necessary. Minor degrees of compensated hemolysis may occur in unoperated patients with severe aortic valve disease.

MICROANGIOPATHIC HEMOLYTIC ANEMIA

Microangiopathic hemolytic anemias are mechanical hemolytic anemias in which the red cell fragmentation is due to contact between red cells and the abnormal intima of partly thrombosed, narrowed, or necrotic small vessels. *In vitro* studies have established the mechanism by which red cells are traumatized as they are forced through a meshwork of fibrin clot. Although parts of the cell membrane are ruptured as the cells are folded around the fibrin strands, the membrane is resealed when the cells escape, and characteristic fragmented cells are formed. Further experimental evidence has correlated fibrin deposition within arterioles and capillaries (i.e. microangiopathy) with the appearance of fragmented red cells (Table 8.9).

Table 8.9: Mechanisms leading to development of drug-related antibodies

Mechanism	*Prototype drugs*	*Type of immuno-globulin*	*Direct Coombs' test*	*Biologic results*	*Frequency of hemolysis*
Immune complex formation (Innocent bystander)	Quinidine phenacetin, stibophen, PAS, Sulfas, thiazide, chlorpromazine, isoniazid, antazoline	IgM or IgG	Positive (often to complement fragment only: however, IgG may be present)	Eluate often negative	Small doses of drug may cause acute intravascular hemolysis with hemoglobinemia, and hemoglobinuria, renal failure is common

Contd.

Table 8.9: Mechanisms leading to development of drug-related antibodies

Mechanism	*Prototype drugs*	*Type of immuno-globulin*	*Direct Coombs' test*	*Biologic results*	*Frequency of hemolysis*
Drug adsorption (Hapten)	Penicillins cephalosporins streptomycin	lgG	Positive (strongly) due to lgG sensitization	Eluate often negative	3–4% of patients on large doses (10 million units) daily of penicillin, which is one of the most common causes of drug, induced immune hemolysis, usually extravascular in nature
Membrane modification (non-immuno-logic protein adsorption)	Cephalosporins	Numerous plasma proteins (non-immunological sensitization)	Positive due to a variety of serum proteins	Eluate negative	No hemolysis, however, 3% of patients receiving the drug develop a positive direct Coombs' test
Methyldopa-induced (unknown)	Methyldopa (Aldomet)	lgG	Strongly positive (due to lgG sensitization)	Eluate positive (warm auto-antibody) identical to antibody found in WAIHA	0.8% develop a hemolytic anemia that mimics a WAIHA (depends on the drug dosage); 15% of patients on Aldomet develop a + direct Coombs' test

HEMOLYTIC UREMIC SYNDROME

The combination of three features, *viz*. acute renal failure, thrombocytopenia, and hemolytic anemia in infants and young children was first described in 1955. A similar, if not identical, disorder occurs in adults, particularly postpartum females and patients undergoing therapy with cyclosporin and various chemotherapeutic agents. In children, its occurrence is usually sporadic, but some familial cases have been recorded. The pathogenesis is uncertain; the initiating event is thought to be damage to endothelial cells in the glomerular capillaries and renal arterioles, resulting in focal platelet clumping and microvascular coagulation and occlusion. The presence or absence of plasma factors that modify platelet behavior have been shown in a few cases.

Clinical Picture

Formerly healthy infants and young children of both sexes are affected, particularly those between 5 and 12 months of age. Diarrhea, vomiting, and pyrexia are the presenting features in most cases. Within 5–14 days, pallor and mild jaundice develop, and the child becomes oliguric. The urine is dark and contains protein, red cells, casts, and sometimes hemoglobin. Skin and mucous membrane bleeding may occur. The spleen is often palpable. Symptoms and signs of uremia and cardiac failure develop, and in occasional cases there is evidence of nervous system involvement. Hypertension is present in about half the patients.

Hematological Picture

The anemia is often severe and is accompanied by a neutropenia and thrombocytopenia of varying, and sometimes severe degree. The blood film shows fragmented red cells

and some microspherocytes. There is reticulocytosis, hemoglobinemia, and moderate hyperbilirubinemia, and the direct antiglobulin test is negative. Laboratory evidence of disseminated intravascular coagulation is present in some patients, but in others coagulation factors are normal or increased. The plasma urea level often reaches 50 mmol/1 or more.

Therapeutics and Prognosis

The prognosis is bad, and mortality in adults is high. Death usually results from acute renal failure. More recently, results of treatment in children appear to be improving, and mortality rates of less than 10% have been reported. Renal failure and hypertension are treated by standard measures, and transfusions are given as symptomatically required. There is no consensus on the value of anticoagulants, fibrinolytic therapy, and antiplatelet agents. Good results have been obtained with plasmapheresis and simple infusion of plasma.

THROMBOTIC THROMBOCYTOPENIC PURPURA AND DISSEMINATED INTRAVASCULAR COAGULATION

Hemolytic anemia with red cell fragmentation is a frequent feature of thrombotic thrombocytopenic purpura. The anemia is often severe with reticulocytosis, hyperbilirubinemia, and normoblastemia. Red cell fragmentation also occurs in some patients with disseminated intravascular coagulation (DIC).

DISSEMINATED CARCINOMA

Microangiopathic hemolytic anemia can occur as an occasional complication of metastatic carcinoma, and may be a presenting symptom. Mucin-secreting carcinomas of breast and stomach are the most common tumors involved, but the condition also occurs with carcinomas of pancreas, lung, and prostate. The anemia, which is moderate to severe, usually has an abrupt onset, and there is often an associated thrombocytopenia. Fragmented red cells (schistocytes) are present, and there is evidence of intravascular hemolysis. Normoblasts and immature granulocytes may also be noted. Clinical and laboratory features of disseminated intravascular coagulation is often present. The fragmentation is thought to be due to the forcible passage of red cells through small vessels containing embolic tumor cells or fibrin deposits. Treatment of the tumor may result in cessation of the hemolysis, and the use of heparin is appropriately justified in some cases to tide the patient over the period of tumor therapy.

MALIGNANT HYPERTENSION AND ECLAMPSIA

Schistocytosis (red cell fragmentation) is a common finding in malignant hypertension, and may be associated with severe intravascular hemolysis and thrombocytopenia. The fragmentation is thought to be due to red cell damage resulting from arteriolar fibrinoid necrosis or intravascular coagulation, evidence of the latter being present in some patients. Severe microangiopathic hemolytic anemia also occasionally occurs is eclampsia.

MARCH (EXERTIONAL) HEMOGLOBINURIA

This is a rare disorder in which hemoglobinemia and hemoglobinuria follow exercise, classically in the upright position, notably on walking, marching, and running. The majority of patients are healthy, young, adult males, often soldiers or athletes who complain of

the passing of red urine after marching or running, respectively. It may also occur in squash players. Hemoglobinuria usually lasts for several hours after the exertion, but in rare cases it persists for several days. It is often the only symptom but sometimes is accompanied by mild constitutional symptoms, e.g. nausea, abdominal cramps, aching in the back or legs, especially the thighs, or a burning feeling in the soles of the feet; these symptoms are usually relatively mild. The degree of hemolysis is seldom sufficient to cause anemia.

The hemoglobinuria is due to traumatic intravascular hemolysis related to a mechanical effect on the blood within the vessels of the soles of the feet. The wearing of resilient insoles may be of value in prevention. A similar syndrome is seen in practitioners of karate who frequently strike hard surfaces on their hands.

HEMOLYTIC ANEMIA ASSOCIATED WITH BACTERIAL INFECTIONS AND PARASITIC INFESTATIONS

The anemia associated with bacterial infections is usually due to bone marrow depression, but red cell survival studies have revealed that a hemolytic factor contributes in some cases. However, infection with certain organisms, notably *Clostridium welchii,* results in frank hemolytic anemia. Malaria is the classical example of hemolytic anemia due to parasitic infestation.

CLOSTRIDIUM WELCHII INFECTION

Clostridium welchii infection regularly causes hemolytic anemia, probably due to the direct action of toxin on the red cells. Most infections are due to postabortal or puerperal infections. The severity of the hemolysis varies, but in cases of *C. welchii* septicemia it is usually marked. With severe infections, the clinical picture is of an acutely ill-patient with features of toxemia and intravascular hemolysis. The anemia is rapidly progressive and severe, with extreme spherocytosis, an increased osmotic fragility, and leucocytosis, the reticulocyte count may not be markedly increased.

MALARIA

Anemia is often found in malaria; it is often only mild or moderate but is occasionally severe, especially with falciparum infections. Its pathogenesis is complex and probably differs at different stages of the illness. Although the degree of anemia often correlates poorly with the extent of red cell parasitization, destruction of parasitized cells in the circulation or spleen is an obvious factor, and a fall in hemoglobin offer follows a chill. Depression of marrow erythropoiesis, dyserythropoiesis, hypersplenism, and immunological damage may also be involved in some patients. A mild leukopenia is usual, although leukocytosis may occur in association with fever and chills. The serum bilirubin may be raised. Diagnosis is established by demonstration of the parasites in the peripheral blood; they appear greatest in number at the time of the chill and in the following 6 hours. Failure to demonstrate the parasite does not exclude the diagnosis, especially in subsiding or chronic cases, and repeated examinations of thick blood films may be necessary. However, with the availability of immunochromatographic techniques the diagnostic aspects have improved considerably. The blood picture returns to normal after cure of the infection. In case of 'chronic' malaria, persistent anemia and splenomegaly are usual.

Plasmodium falciparum has a rare but serious complication–Blackwater fever and is characterized by an acute hemolytic anemia with marked hemoglobinuria. Blackwater fever occurs chiefly in tropical and subtropical regions where malaria is endemic. There is no racial immunity, but Europeans, usually persons who have had repeated malaria attacks, are commonly affected. It frequently seems to be precipitated by the taking of antimalarial drugs, usually quinine, especially when the latter is taken irregularly for suppression or treatment.

LEAD POISONING

Hematological alterations, particularly basophil stippling of the red cells, are commonly present in persons exposed to lead. Stippling is now generally considered to be an unreliable criterion of lead intoxication, since the number of stippled cells does not correlate well with the intensity of exposure; occasionally, stippling is absent in patients with lead poisoning. Further, stippled cells can be found in increased numbers in a variety of hematological disorders, e.g. various hemolytic anemias, leukemia, and after exposure to other industrial toxins. Most cases of lead poisoning occur in persons working in lead industries. However, a certain number of non-occupational cases occur, most commonly in children, usually from the chewing of lead toys, furniture, or other articles painted with lead paint.

Anemia is usual in lead poisoning. It is usually of mild-to-moderate severity, hemoglobin values only rarely falling below 9 g/dL.

The red cells are characteristically normocytic and normochromic, or microcytic and hypochromic, and may show mild polychromasia. A slight reticulocytosis is common.

The bone marrow shows erythroid hyperplasia with 'ring' sideroblasts and thus the anemia of lead poisoning may be classified as a secondary sideroblastic anemia.

The pathogenesis of the anemia of lead poisoning is not fully understood; it appears that the main factor is an interference with marrow hemopoiesis, but that there is also some hemolytic element, probably due to direct red cell membrane damage. There is evidence of inhibition of enzymes involved in heme synthesis; this is reflected in the increased free erythrocyte protophyrin and increased excretion of coproporphyrin and delta-aminolaevulinic acid (ALA) in the urine. Globin synthesis is also impaired (Fig. 8.17)

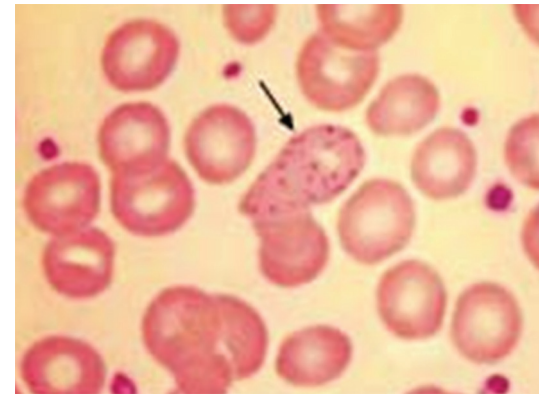


Fig. 8.17: Lead poisoning, peripheral smear

BURNS

Hemoglobinuria frequently occurs in severely burnt patients. It has been shown that heating of red cells to a temperature above 51°C results in spherocytosis with consequent increased osmotic and mechanical fragility, and in red cell fragmentation. The cells passing through the burnt area at the time of the burns are thus irreversibly damaged and are destroyed intravascularly, with resultant hemoglobinemia and hemoglobinuria.

SUMMARY OF CLINICAL INVESTIGATIONS

Given below is the summary of the clinical investigations that should be carried out in a patient with suspected hemolytic anemia:

Summary of the investigation of a patient with suspected hemolytic anemia

- Personal and medical history related aspects
- Age and sex
- Age at onset of symptoms
- Race to which the person belongs
- Occupation
- Jaundice; color of urine and feces
- Crises
- Cholelithiasis
- Family history (anemia, jaundice, splenomegaly, dark urine, cholelithiasis)
- Hemoglobinuria; relation to sleep, cold, exercise, or drugs
- Symptoms suggestive of disorders causing secondary AIHA
- Raynaud's phenomenon
- Drug, chemical or alcohol ingestion
- Recent travel to tropics.

PHYSICAL EXAMINATION

- Pallor, cyanosis
- Jaundice, splenomegaly, hepatomegaly
- General development, facies, presence of congenital abnormalities, leg ulceration, or pigmentation (hereditary hemolytic anemia)
- Signs of disorders causing secondary AIHA, especially lymph node enlargement and purpura
- Special investigations.

MANDATORY INVESTIGATIONS FOR ALL CASES

Full blood examination, with special reference to:

- Morphology of red cells in a well-made and well-stained blood film (note especially spherocytosis, autoagglutination, fragmentation, inclusion bodies)
- Reticulocyte count
- Plasma haptoglobin
- Serum bilirubin
- Osmotic fragility test
- Direct antiglobulin test
- Examination of urine for urobilinogen, hemoglobin, hemosiderin.

FURTHER INVESTIGATIONS NECESSARY IN SOME CASES

- Bone marrow aspiration and trephine biospy (evidence of lymphoma, folate deficiency in AIHA)
- Measurement of red cell life span (establish hemolytic nature in doubtful cases)
- Examination of plasma for hemoglobin and methemalbumin (intravascular hemolysis)
- X-ray of skull, hands, and long bones (hereditary hemolytic anemia)
- Sickle test (sickle-cell anemias)

Tests for abnormal hemoglobins:

- Hemoglobin electrophoresis
- Alkali denaturation
- HbH inclusions
- Heat instability test
- Investigation of relatives (hereditary hemolytic anemia)
- Examination of red cells for methemoglobin and sulfhemoglobin (chemical hemolytic anemia, hereditary hemolytic anemia)
- Estimation of red cell G6PD and other enzymes (hereditary hemolytic anemia, chemical hemolytic anemia)
- Cold agglutinins (AIHA)
- Investigations to demonstrate etiology in secondary AIHA, especially LE cell test and lymph node biospy
- VDRL test (paroxysmal cold hemoglobinuria)
- Donath–Landsteiner test (paroxysmal cold hemoglobinuria)
- Ham's acid serum test, sucrose hemolysis test

CHAPTER

9

Disorders of Hemoglobin Structure and Synthesis

The hereditary disorders of hemoglobin may be classified into two broad groups, the hemoglobinopathies and the thalassemias. The hemoglobinopathies are characterized by the production of structurally defective hemoglobin due to abnormalities in the formation of the globin moiety of the molecule. The thalassemias are characterized by a reduced rate of production of normal hemoglobin due to absent or decreased synthesis of one or more types of globin polypeptide chains.

Thalassemias are widespread, with maximum prevalence around the Mediterranean littoral and in Southeast Asia. The common abnormal hemoglobins, HbS and HbC are prevalent in tropical Africa and among Black populations in the New World. HbE is common in Southeast Asia, HbD-Punjab in the Indian continent. Hereditary disorders of hemoglobin are less common among people of Northern European origin, but no ethnic group is totally spared.

NORMAL HEMOGLOBIN

Hemoglobin is a conjugated protein of molecular weight 64000, consisting of two pairs of polypeptide chains to each of which a heme is attached. Human hemoglobin exists in a number of types, which differ slightly in the structure of their globin moiety. However, the heme is identical in all types.

HEMOGLOBIN TYPES

Hemoglobin A (HbA) comprises about 97% of the hemoglobin of adult red cells. It consists of two alpha (α) and two beta (β) chains with the structural formula $\alpha_2\beta_2$. The α chain contains 141 amino acids, and the β chain, 146. Small amounts of HbA are detected in the fetus as early as the eighth week of life. During the first two months of postnatal life, HbA almost completely replaces HbF, and the adult pattern is fully established by six months.

Hemoglobin A_2 (HbA_2) is the minor hemoglobin in the adult red cell. It has the structural formula of $\alpha_2\delta_2$, the delta (δ) chain containing 146 amino acids. The α chain is identical to that of HbA. HbA_2 is present in very small amounts at birth and reaches the adult level of 1.5–3.2% during the first year of life. Elevation of HbA_2 is a feature of some types of thalassemia and occasionally occurs in megaloblastic anemia and unstable hemoglobin disease. HbA_2 may be reduced in iron deficiency (Table 9.1).

Hemoglobin F (HbF) is the major respiratory pigment from early intrauterine life up to term. It has the structural formula $\alpha_2\gamma_2$, each gamma (γ) chain consisting of 146 amino acids. The γ chains are designated $^{G}\gamma$ or $^{A}\gamma$ depending on whether they have glycine or

alanine at position 136. At term, HbF accounts for 70–90% of the total hemoglobin. It then falls rapidly to 25% at 1 month, and 5% at 6 months.

Table 9.1: Normal human hemoglobins

	Hemoglobin type	*Structural formula*
Adult	HbA	$\alpha_2\beta_2$
	HbA_2	$\alpha_2\delta_2$
Fetal	HbF	$\alpha_2\gamma_2$
	Hb-Barts	γ_4
Embryonic	Hb-Gower 1	$\zeta_2\varepsilon_2$
	Hb-Gower 2	$\alpha_2\varepsilon_2$
	Hb-Portland	$\zeta_2\gamma_2$

The adult level of about 1% is not reached in some children until puberty. HbF is elevated in some hemoglobinopathies and thalassemia syndromes. It may be elevated in occasional cases of congenital and acquired aplastic anemia, megaloblastic anemia, paroxysmal nocturnal hemoglobinuria, sideroblastic anemia, and in some forms of leukemia. It is also occasionally raised in early pregnancy. The acid elution test indicates that HbF is unevenly distributed in the red cells in these conditions. HbF is measured by the alkali denaturation technique.

Hb-Gower 1 and Hb-Gower 2 are confined to the embryonic stage of development. They contain epsilon (ε) and zeta (ζ) chains, Hb-Gower 1 being $\zeta_2\varepsilon_2$ and Hb-Gower 2 being $\alpha_2\varepsilon_2$. Hb-Portland is found in trace amounts throughout intrauterine life and in neonates. It has the structural formula $\zeta_2\gamma_2$. Hb-Bart is also found in small amounts in cord blood if sensitive techniques are used. Both Hb-Portland and Hb-Bart are increased in the cord blood of neonates with α-thalassemia.

HEMOGLOBIN STRUCTURE

The structure of the hemoglobin molecule may be viewed at four levels of organizational complexity. The basic arrangement of linked amino acids forming four polypeptide chains, each attached to a heme molecule, is the primary structure. Each chain is arranged in a series of eight helical segments joined by short non-helical segments, 8% of the total length of each chain is in helical conformation, and this is referred to as the secondary structure.

The folding of each coiled chain into a specific three-dimensional configuration is the tertiary structure. The four-folded chains fit closely together to form a compact tetrameric molecule known as the quaternary structure. Each heme molecule is enclosed in a pocket by the folds of the chain. The integrity of the chains and their spatial relationships to each other and to the heme molecules are critical in the maintenance of stability of the molecule and its ability to transport oxygen.

GENETIC REGULATION

Every person receives one or more genes from each parent for each of the four major hemoglobin chains. β- and δ- chain syntheses are each under the control of single genes, but the gene loci of α and γ chains are duplicated.

Recent advances in molecular biology have greatly increased our knowledge of the fine structure of the human globin gene complex. The chromosomal organization of the α and β gene complexes is depicted in Fig. 9.1. The α gene complex is situated on the short

arm of chromosome 16 and includes the duplicated α genes (α_1 and α_2), a non-functional pseudo-α gene ($\psi\alpha_1$) and two ζ genes, one of which is probably non-functional. The non-α gene complex is situated on the short arm of chromosome 11 and includes the β and δ genes, the duplicated γ genes ($^{G}\gamma$ and $^{A}\gamma$), the e gene and a non-functional pseudo-β gene ($\psi\beta_1$).

Synthesis: The genetic information that directs the synthesis of individual globin chains in erythroblasts is encoded in the nucleotide base sequence of the corresponding gene DNA, each triplet codon of three nucleotide bases specifying a single amino acid. Each gene consists of regions (referred to as exons) that code for globin messenger RNA (mRNA) and other non-coding 'intervening sequences' (introns). Within the nucleus, the gene is transcribed into a large mRNA precursor from which the transcripts of the 'intervening sequences' are then removed by an enzymatic process called splicing. The modified mRNA moves to the cytoplasm where it combines with ribosomes and is translated into the globin chain.

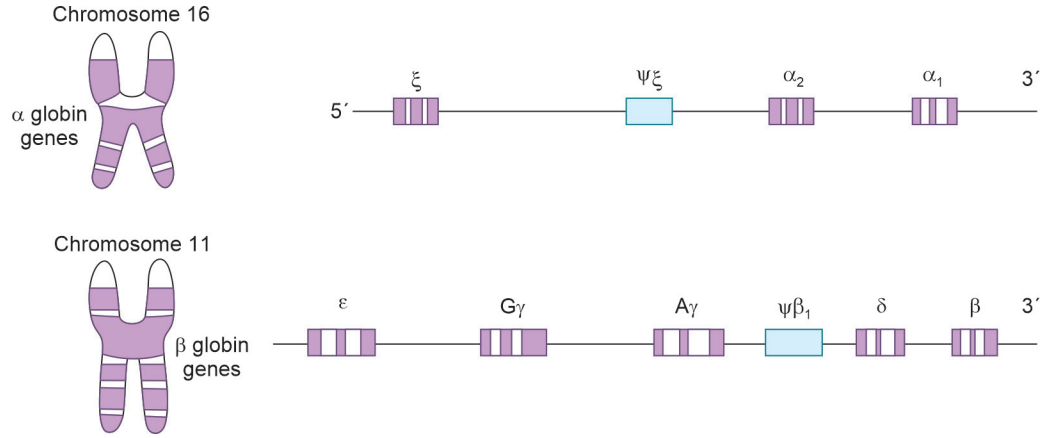


Fig. 9.1: Structural organization of the human globin genes. Globin genes related to a globin are located in chromosomes number 16, and their sequence in the DNA strand from embryonic (ζ) through ineffective 'pseudogene' forms (ψ) to the two distinct adult (α) genes is illustrated. The corresponding sequence of the β-related globin genes on chromosomes 11 from the embryonic (ε) through fetal (γ) and δ to adult β globin genes is also illustrated

ABNORMAL HEMOGLOBINS AND THE HEMOGLOBINOPATHIES

Most of the abnormal hemoglobins have been described. Each arises from a mutation affecting the gene directing the structure of a particular pair of polypeptide chains, and they are classified as α-, β-, γ- or δ-chain variants depending on the chains involved. The mutant gene is situated at the same chromosomal locus as (i.e. is an allele of) the normal gene controlling production of the corresponding normal chain.

When the possession of a hemoglobin variant gives rise to a clearly defined disease state, the affected person is said to have a hemoglobinopathy. It is important to appreciate, however, that the great majority of abnormal hemoglobins confer no harmful effect, and the individual remains asymptomatic and unaware of the abnormality within the red cell.

GENETIC REGULATION

Abnormal hemoglobins are inherited as autosomal co-dominants. Thus, subjects who inherit one normal and one abnormal gene are heterozygotes, and those who have two identical abnormal genes are homozygotes. Double heterozygotes are subjects who have inherited two different abnormal genes. The homozygous state is usually referred to as

the 'disease' (e.g. the homozygous state for HbC is 'HbC disease'), and the heterozygous state as the 'trait' (e.g. 'HbC trait'). This rule has some exceptions, however, e.g. unstable hemoglobin 'disease' is a reflection of a heterozygous state.

Each group of chain variants and the disorders associated with them have some common characteristics.

Beta-chain variant hemoglobins: Beta chains take part in the formation of HbA only, and thus β-chain variants are all variants of HbA. Heterozygous subjects synthesize both normal and abnormal β chains, and the abnormal hemoglobin is usually about 30–40% of the total. Homozygous subjects synthesize the abnormal hemoglobin and the normal small amounts of HbA_2, but no normal β chains and thus no normal HbA. Heterozygotes for two β-chain variants have equal amounts of the two abnormal hemoglobins and a small amount of HbA_2 in their red cells. As β-chain synthesis commences in intrauterine life, β-chain variants may be detected in the fetus. Clinical effects from the abnormal hemoglobin do not occur until after birth when γ-chain synthesis falls to a low level. The majority of abnormal hemoglobins are β-chain substitutions, and about 200 such variants have been described.

Alpha-chain variant hemoglobins: Alpha chains are involved in the formulation of HbA, HbA_2, and HbF, and thus α-chain substitutions affect all these hemoglobins. Adult heterozygotes for α-chain variants produce both normal and abnormal HbA, HbF and HbA_2, the abnormal types having abnormal α chains in addition to the normal $\alpha_1\gamma$ and δ chains. The major hemoglobin variant (the HbA variant) ranges from 15 to 45% of the total hemoglobin in the red cell. About 100 α-chain variants have been described.

TYPES OF STRUCTURAL ABNORMALITIES (TABLE 9.2)

a. Amino acid substitution
   - Single
   - Double

b. Abnormal hemoglobin with long chains

c. Intermixing of various chains, e.g. Hb-Lepore
   - HbS $\alpha_2\beta_2$ 6 glu → val (at position 6, glutamine is replaced by valine)
   - HbC $\alpha_2\beta_2$ 6 glu → lys
   - HbE $\alpha_2\beta_2$ 26 glu → lys
   - HbD (Punjab) $\alpha_2\beta_2$ 121 glu → gln

Table 9.2: Common abnormal hemoglobins

Hemoglobin	*Structural formula*
HbS	$\alpha_2\beta_2^{\,6\ glu \rightarrow val}$
HbC	$\alpha_2\beta_2^{\,6\ glu \rightarrow lys}$
HbE	$\alpha_2\beta_2^{\,26\ glu \rightarrow lys}$
HbD-Punjab	$\alpha_2\beta_2^{\,121\ glu \rightarrow gln}$

LABORATORY DIAGNOSIS

Although exact identification of an abnormal hemoglobin usually requires an array of special biochemical methodologies, the initial investigation of the hemoglobinopathies is well within the scope of most routine clinical laboratories. The clinical findings, the patient's ethnic origin and family history, and preliminary hematological studies suggest the diagnosis, and hemoglobin electrophoresis demonstrates the presence of an abnormal

hemoglobin. Other simple laboratory tests based on physicochemical properties of some abnormal hemoglobins, e.g. the sickle test, may permit a presumptive diagnosis at this point. Final definitive identification of the abnormal hemoglobin usually requires the assistance of a reference laboratory.

Routine Hematological and Biochemical Tests

Essential initial diagnostic tests are determination of the hemoglobin level, packed cell volume, red cell count and red cell indices, preferably by means of an electronic cell counter, together with a reticulocyte count and examination of a stained blood film by an experienced observer. Bilirubin estimation and other biochemical tests for the presence of hemolysis should also be performed.

Investigations Depending on Physicochemical Properties of Abnormal Hemoglobins

Tests of this type include the sickle test, the hemoglobin solubility (HbS) test, the demonstration of intracellular hemoglobin crystals (HbC), HbH inclusions (α-thalassemia) and Heinz bodies, the heat instability test and isopropanol precipitation test (the unstable hemoglobins), and oxygen dissociation studies (the high oxygen-affinity hemoglobins).

Hemoglobin Electrophoresis

This is the most useful method for the demonstration of abnormal hemoglobins. The hemoglobins are separated on a variety of supporting media on the basis of electric charge differences. Cellulose acetate electrophoresis at pH 8.6 is the method of choice in most clinical laboratories. Agar gel electrophoresis using a citrate buffer at pH 6.0 is useful in supplementing (but not replacing) the information gained from other methods, as the mobility of some abnormal hemoglobins on agar gel differs from that on other supporting media. The electrophoretic mobility of the commonly encountered normal and abnormal hemoglobins on cellulose acetate and agar gel is schematically depicted in Fig. 9.2.

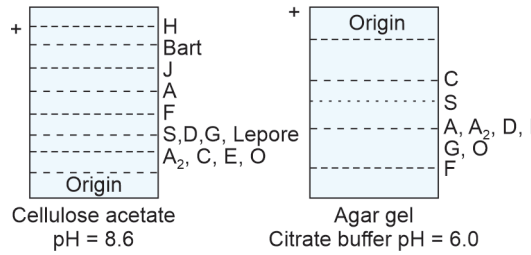


Fig. 9.2: Hb electrophoresis

- Clinical findings
- Ethnic origin of patient
- Any family history
- Preliminary blood studies

→ Suggest hemoglobinopathy → Hb electrophoresis confirms

Special tests:

- Tests depending on physicochemical properties
- Sickle test, Hb solubility test—HbS
- Intracellular Hb crystals—HbC
- HbH inclusions—α-thalassemia
- Heinz bodies
- Heat instability test
- Isopropanol precipitation test: Unstable Hb
- Oxygen dissociation studies (high O_2 affinity Hb)

Alkali Denaturation

HbF is resistant to denaturation by alkali, and in clinical practice is estimated by the alkali denaturation technique. This test measures the percentage of alkali-resistant pigment remaining after exposure to alkali under standard conditions. Values over 2.5% in adult subjects are regarded as elevated.

The Acid Elution Test

Red cells containing HbF resist elution at an acid pH to a greater extent than do normal cells containing HbA. The acid elution makes us of this phenomenon to permit the cytochemical assessment of the HbF content of individual cells.

Hemoglobin Electrophoresis

Even though the sickle and hemoglobin solubility tests detect the presence of HbS, hemoglobin electrophoresis is mandatory for precise diagnosis of the sickle hemoglobinopathies. HbS may be demonstrated by electrophoresis on cellulose acetate at pH 8.6 in a position between HbA and HbA_2. Although nearly 50 hemoglobin variants have a similar mobility to HbS, the only practical problem of any frequency caused by identical mobilities is the differentiation of homozygous sickle-cell disease from sickle-cell HbD disease. Red cells from patients with both conditions sickle, and as HbS and HbD migrate in the same position on cellulose acetate, the two diseases appear to be identical. The problem may be resolved by electrophoresis in agar gel at pH 6.0. On this medium, HbS and HbD separate widely; thus, homozygous sickle-cell disease.

A two-band pattern on agar gel would confirm sickle-cell HbD disease, and a one-band pattern homozygous sickle cell disease.

Antenatal diagnosis of the sickle hemoglobinopathies is now possible.

SICKLE HEMOGLOBINOPATHIES

These are hereditary disorders in which the red cells contain HbS. They include the heterozygous (sickle-cell trait) and the homozygous (SS disease) states for HbS, and conditions in which HbS is combined with other hemoglobin structural variants or thalassemia. In the deoxygenated state, the solubility of HbS is 10% of that of HbA. The conformational changes in HbS induced by deoxygenation cause the cells containing the abnormal hemoglobin to become rigid and deformed, assuming a sickle or crescent shape.

The red cell sickling in the circulating blood has two major pathological effects:

i. The distorted and rigid cells block small blood vessels, impairing flow and causing ischemia and infarction; and
ii. Repeated 'sickle-unsickle' cycles lead to loss of fragments of red cell membrane, and the cells become spherocytic and fragile. They are removed prematurely by the reticuloendothelial system, and to a lesser extent destroyed in the circulation resulting in both extravascular and intravascular hemolysis.

HbS differs from HbA in the substitution of valine for glutamic acid in the sixth position from the N-terminal end of the β chain. The precise mechanism by which this seemingly minor change in amino acid sequence leads to such an important rearrangement of the molecule on deoxygenation is not known with certainty. Electron microscopy of sickle cells has shown bundles of long tubular fibrse parallel to the long axis of the cell which are presumed to be of sufficient rigidity to distort the cell membrane. Each fiber consists of 14 or possibly 16 spirally-wound filaments, the filaments being composed of HbS molecules like beads on a string.

Red cells containing large amounts of HbS begin to sickle at an oxygen tension of 50–60 mmHg. This tension is experienced by the cells in parts of the microcirculation, and thus sickling occurs *in vivo.* If the flow rate is rapid, sickling does not become fully established and the cells resume normal shape when they are swept back to areas of circulation where the oxygen tension is higher. If the flow rate is slow, and the cells are delayed in areas where the oxygen tension is low, the cells sickle and there is further slowing of the circulation. Oxygen tension is further reduced, additional sickle cells accumulate, and finally complete blockage of the vessel occurs. Tissue ischemia resulting from such vessel blockage is the basis of the painful crises that are a major clinical feature of the sickling disorders.

Several factors influence the degree of deoxygenation required to produce sickling of red cells containing HbS.

- The amount of HbS in the red cell is clearly of importance as the cells of a patient with sickle-cell trait which contain less than 50% HbS are less likely to sickle at a particular level of deoxygenation than the cells of a patient with homozygous sickle cell disease which contain nearly 100% HbS.
- The physical properties of the hemoglobin with which HbS is associated in the red cell may increase or decrease the liability of the HbS to sickle. HbC and HbD potentiate sickling, and patients heterozygous for these hemoglobins and HbS have moderately severe sickling disorder. HbF has the opposite effect and tends to diminish sickling.

Red cells of patients with homozygous sickle cell disease have a reduced oxygen affinity, and the oxygen dissociation curve of the blood is shifted to the right. Although this phenomenon assists the release of oxygen at the tissue level, it also results in the occurrence of sickling at a higher oxygen tension than would be the case if the dissociation curve was normal. Acidosis shifts the curve further to the right and similarly enhances the sickling process.

The packed cell volume and proportion of red cells containing HbS are also important in determining the increase in blood viscosity resulting from a fall in oxygen tension.

The sickle gene is the result of a point mutation in the codon for the sixth amino acid of the β globin chain, and is inherited as a mendelian co-dominant. It occurs mainly in blacks or persons with an admixture of black blood, and is thus seen frequently in Africa and amongst black populations in north and south America and the West Indies. It is also found in certain regions in Greece, southern Italy, Turkey, the middle East, and India. The sickle gene is believed to confer some protection against Plasmodium falciparum malaria, and its geographical distribution is in accordance with this concept. The following sickle hemoglobinopathies are prevalent in communities where the sickle gene is found–sickle-cell trait, homozygous sickle-cell disease, sickle-cell thalassemia, and sickle-cell HbC disease.

Sickle Hemoglobinpathies include

- Sickle cell disease (SS) } Homozygous
- Sickle cell trait (AS) }
- HbS-C } Heterozygous
- HbS-Thal. }

- Homozygous
- In the deoxygenated state solubility of HbS is 1/40th of deoxygenated HbA
- Confirmational changes are caused by deoxygenation called tactoid formation, i.e. sickling

- Sickling
  - Hyperviscosity → blocks microcirculation → infection
  - Rapid sickle—unsickle → loss of cell membrane

Early removal by RES and to some extent intra- and extravascular hemolysis ← ←RBC spherocytosis and fragility

- Electron microscopically: Sickle cells show parallel fibers.
- Each fiber has 6 spirally wound filaments around a core. Filaments of HbS 'molecules are like beads on a string.
- RBCs with HbS will sickle at O_2 tension of 50–60 mm of Hg (in microcirculation → tissue ischemia *in vivo*)

↓

painful crises of sickling

Factors for sickling:

1. Amount of HbS <50% AS → less liable → 100% SS → easily sickled
2. Association with other hemoglobinopathies, e.g.

HbS-C, HbS-D } Potentiate sickling

Patients' heterozygotes for these have severest sickling disorders

3. HbF prevents sickling
4. O_2 dissociation curve shifted to right in SS, although this assists release of O_2 at the tissue level but it also increases sickling at higher O_2 tissue tension. Acidosis further shifts the curve to right.

Laboratory Diagnosis

1. **Sickle test:** HbS cells sickle on being mixed with freshly prepared sodium meta-bisulfite false + test may occur in (Fig. 9.3):
   - Patients who have received recent blood transfusion,
   - Infected blood sample,
   - Old reagent
2. **Hb solubility test:** Reduced HbS is relatively insoluble in concentrated phosphate buffer. Hb added to a solution of sodium dithionite (a reducing agent) containing a phosphate buffer, development of turbidity implies presence of HbS.

   Whole blood can be used in this test but saponin (a lysing agent) has to be added. False + tests may occur in:
   - Neonates
   - Abnormal plasma proteins (if whole blood is used),
   - If RBCs contain Heinz bodies
   - PCV too high or too low
   - Old reagents

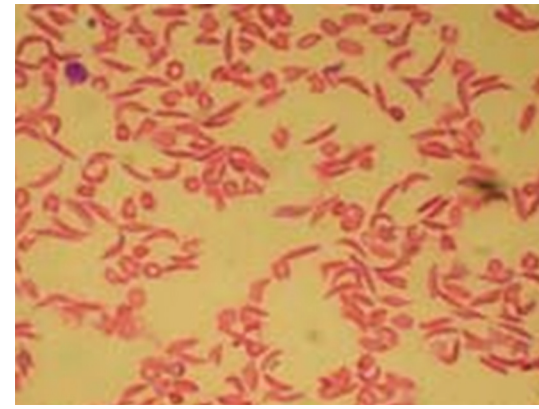


Fig. 9.3: Sickled cells after addition of reducing agent

3. **Hb electrophoresis is mandatory:** Cellulose acetate (pH = 8.6) is good but if HbD is coexisting: S and D separation would not occur, in that case use agar gel (pH = 6.0) with citrate buffer (Table 9.3).

Table 9.3: HbS disorders—electrophoresis phenotypes				
Disorder	*A%*	*A2%*	*F%*	*S%*
AS	55–70	2–4	Normal	38–45
SS	0	2–5	1–20	75–95
HbS-β-thalassemia S-β^+ (some β chains are present)	10–30	4–8	2–10	60–85
S-β_2 (no β_2 chains)	0	4–8	5–30	70–90
HbS-C (SC)	0	35–50 ($C+A_2$)	1–5	50–55
HbS-D(SD)	0	Normal	1–5	95 (S+D)
Sickle cell trait or α-thalassemia trait	65–75	N	N	20–30

SICKLE CELL TRAIT (AS)

Sickle cell trait, the asymptomatic carrier state for HbS, occurs in about eight percent of American blacks. In Africa, its prevalence rate in many populations is over 20%, and reaches 50% in some tribes. Sickle cell trait is the heterozygous state for the HbS gene. HbS comprises 38–45% of the total hemoglobin, the rest being HbA, HbA_2 and HbF. The cells do not contain sufficient HbS to undergo sickling at the lowest oxygen tension normally occurring in the body, and the red cell lifespan is normal.

In the stained blood film, no sickle cells are present and the red cells appear normal. The MCV and MCH are also normal. However, sickling can readily be demonstrated by the sickle test, and the hemoglobin solubility test is positive. If the patient has concurrent α-thalassemia, the red cells are microcytic with a mild reduction in MCV and a Hb-S concentration of <38%.

Sickle cell trait does not cause anemia, and in general is asymptomatic. If anemia is present, other causes, e.g. iron deficiency, should be sought. A high proportion of affected subjects show defects in urine concentrating ability, and hematuria is an occasional complication. Rare episodes of splenic infarction during flight at high altitude in non-pressurized aircraft have been described, and severe hypoxia resulting from administration of anesthetic agents and other respiratory depressants may in exceptional cases be associated with *in vivo* sickling and serious thromboembolic sequelae. There are some evidences that sickle cell trait is a risk factor for sudden death during unaccustomed exercise, but most epidemiological studies suggest that no selective morbidity or mortality is attributable to the condition.

Is an asymptomatic heterozygous carrier state
- Rarely associated with clinical or hematologic manifestations of importance
- There is no anemia and red cell morphology is normal
- HbS <50%, normally red cells do not undergo *in vivo* sickling
- However, sickle tests and solubility tests are positive
- Occasional complication is hematuria
- Rarely splenic infarction may occur
  - Flying in unpressurised aircraft
  - Pulmonary infection
- AS protects from falciparum malaria

SICKLE CELL ANEMIA

HbS is enough for *in vivo* sickling, is seen in southern India.

In vivo sickling may cause

- Chronic hemolytic anemia
  - Organ damage
  - Episodes of pain

Clinical

- Diagnosis usually made at age less than 2 years
- From birth to 6 months, HbF protects
- Have increased incidences of infections with
  - Pneumococci
  - Meningococci
  - *Haemophilus*
- Autosplenectomy occurs by 8 years or so
- There is abnormal neutrophilic chemotaxis and phagocytosis
- Hand–foot syndrome: Microinfarction of medulla of carpal and tarsal bones
- Splenic sequestration syndrome: blood pooling in spleen → hypovolemia → shock death.
- Splenomegaly: Evident by 6th month, autosplenectomy occurs by 8 years—so even though spleen is enlarged but changes of splenectomy (functional asplenia), e.g.: Howell-Jolly bodies, target cells, failure of spleen to accumulate radioactive colloidal sulfur, are there.
- In adults: Clinical severity is variable, although all are anemic.

Sickle Cell Crises

Incidence reduces with increasing age.

1. Vaso-occlusive Crises

- Are typically recurrent and potentially catastrophic
- Clinical manifestations are sudden in onset and directly attributable to obstruction of the microcirculation by intravascular sickling
- There is little or no change in hematologic values
- Precipitating factors
  - in adults
    - infections
  - in children
    - fever, dehydration and acidosis.

Sometimes a history of recent exposure to cold may be obtained. Sudden changes in the altitude and travel in nonpressurized aircraft are less common antecedents.

Hand and Foot Syndrome

The initial episode in young children often involves the small bones of hand and feet. The dorsa of hands and feet are swollen, nonerythematous, and very painful. Fever and leukocytosis are common. X-ray changes are limited to soft tissue swelling: Cortical thinning and marked destruction of metacarpals, metatarsals, and phalanges appear 2–3 weeks after the onset of symptoms. It may recur till the patient is about 3 years of age.

Bone and Joint Crises

After about 3 years of age sludging of blood occurs in the larger bones of the extremities, the spine, rib cage and periarticular structures, producing bone and joint crises. Pain results from ischemia of bone, bone marrow, periosteum, or periarticular tissues. It is of gnawing character and progress in severity.

Abdominal Crises

These are attributable to small infarcts of mesentery and abdominal viscera and are characterized by severe abdominal pain and signs of peritoneal irritation.

CNS Crises

Occur in children and young adults. There is sudden occlusion of cerebral vessels. Hemiparesis, aphasia, seizures, sensory deficits and altered sensorium occur single or together. Intracerebral or subarachnoid hemorrhage may result from hypoxic necrosis of vessels. Cerebral angiography demonstrates stenosis and thrombotic involvement of medium and large vessels (in larger vessels the involvement initially is of vasa vasorum).

Pulmonary Crises

Characterized by fever, tachypnea, chest pain, increased leukocytosis, pulmonary infiltrates and pulmonary crises are the single most common expressions of sickle cell anemia requiring hospitalization. These crises may occur alone or with pulmonary infections.

2. Hematologic Crises

Hematologic crises, characterized by sudden exaggeration of anemia, are unrelated to vaso-occlusive crises. If unrecognized, the hemoglobin fall may be so acute as to cause heart failure and death within hours.

Aplastic Crises

Temporary arrest of erythropoiesis usually is associated with or follows a febrile illness. In the early phase of a crisis, peripheral reticulocytes and bone marrow normoblasts disappear or are greatly reduced. The process is self-limited and erythropoiesis usually starts within 5 to 10 days with appearance of reticulocytes and normoblasts in the peripheral blood.

Splenic Sequestration Crises

Characterized by sudden trapping of blood in the spleen. It occurs almost exclusively in infants and young children whose spleens are usually enlarged. The already enlarged spleen rapidly increases in size at the expense of blood volume. Hypovolemic shock and death may occur within hours.

Hemolytic Crises (Hyperhemolytic Crises)

There is sudden acceleration of the hemolytic process. They have been described in association with G6PD deficiency and with hereditary spherocytosis. These crises also occur following challenge by oxidant drugs and infections.

Megaloblastic Crises

These crises result from the sudden arrest of erythropoiesis by folate depletion. Chronic erythroid hyperplasia imposes a drain on folate reserves. Megaloblastic crises are likely to occur when food consumption is interrupted by illness or alcoholism or when folate requirement is further augmented by rapid growth or pregnancy.

3. Infectious Crises

Infection is the major presenting manifestation of sickle cell anemia in early childhood. During the first 5 years of life, *Str. pneumoniae* is the major offending organism and the blood and spinal fluid (meningitis) are major sites of infection. Incidence of *H. influenzae* infection is also higher in these children. After 5 years of age. Gram-negative bacteria replace *Str. pneumoniae* as the major offenders. Osteomyelitis especially due to Salmonella is common in these patients. The pathophysiologic basis for increased susceptibility to aggressive infection is due in large part of the loss of spleen function.

In addition, there are a number of other signs and symptoms:

- Dorsal kyphosis, lumbar lordosis
- Hypogonadism in males
- Conjunctival icterus and pallor
- Tender hepatomegaly (tender microinfarctions)
- Cholelithiasis
- Autosplenectomy
- Cardiomegaly
- Pulmonary fibrosis → pulmonary hypertension

  ↓

  right ventricular failure ← right ventricular hypertrophy
- Plueritic pain
- Brain: Headache, hemiplegia
- Chronic renal failure, fixed low specific gravity urine.
- Renal medullary infarction, papillary necrosis and hematuria
- Eye: Infarction of peripheral retina
- Chronic leg ulcers
- Osteoporosis
- In elderly patients
  - Periosteal thickening
  - Osteosclerosis of long bones
  - Aseptic femoral head necrosis
  - Increased susceptibility to *Salmonella osteomyelitis*
- Sterility in females

Blood Picture

- Anemia
- Moderate to marked: Normochromic (MCV and MCH are normal) anemia
- It occurs because of reduced RBC life span to about 8 days and in part due to ineffective erythropoiesis
- Hemolytic and aplastic crises further reduce Hb.
- Irreversibly sickled cells (ISC have a life of 2 days (Fig. 9.4)
- Osmotic fragility is decreased
- ESR is decreased, sickling prevents Rouleaux formation
- There is moderate to marked anisopoi-kilocytosis
- Serum and red cell folate values are decreased
- There is an evidence of intravascular hemolysis

- Sickle and solubility tests are positive
- There is irregular distribution of HbF (acid elution test)
- Hemoglobin electrophoresis shows increased HbS.

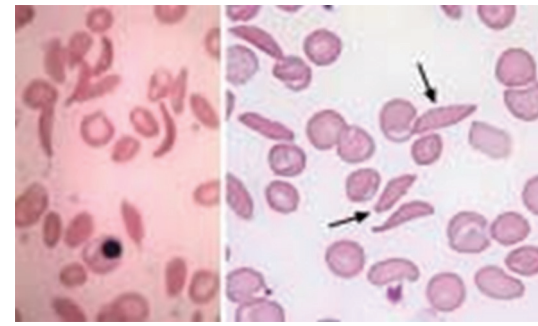


Fig. 9.4: HbSS disease, peripheral smear

Prognosis

Serious in childhood only.

Treatment

- Prophylactic—avoid precipitating factors
- Folic acid—5 mg daily
- Good general nutrition and hygiene
- Crises—rest, rehydrate, antibiotics if infection occurs, bicarbonate if patient becomes acidotic. Transfusion in severe anemia.
- Extra care during pregnancy and anesthesia.

COMBINATION OF HbS WITH OTHER HEMOGLOBINOPATHIES

SICKLE CELL β-THALASSEMIA

Sickle cell β-thalassemia represents the double heterozygous state for the HbS and the β-thalassemia genes. It occurs mainly in persons of Greek and Italian origin, and in Blacks. The clinical and hematological manifestations are highly variable. In general, the condition resembles homozygous sickle cell disease, but tends to be less severe. Much of the variability is ascribed to the existence of two types of sickle cell β-thalassemia, one characterized by a complete absence of HbA due to the presence of β^o-thalassemia gene (see below) and the other HbA levels of 10–30% due to a β^+ gene.

In the Mediterranean region, sickle cell β-thalassemia is usually a relatively severe disorder with little or no HbA in the red cells, an early onset, marked anemia, and a high mortality rate in childhood. Painful crises, the hand–foot syndrome, and aseptic necrosis of bone all occur, and hepatosplenomegaly is usual. Some patients are less severely affected and reach adult life without major symptoms.

In Blacks, the condition is milder, and red cells contain 10–30% HbA. Many patients have a little disability and may be detected by a chance hematological screening examination. Some have occasional painful crises, but lead a normal life otherwise. A small proportion has a more serious illness, similar to that seen in Greek and Italian subjects. Splenomegaly is present in about 50% of patients regardless of clinical severity.

The blood picture in sickle cell β-thalassemia is similar to that of β-thalassemia major. The hemoglobin level in the β^o type is 6–9 g/dL, and in the β^+ type 10–11 g/dL. Microcytosis, marked hypochromia, and the target cells are the main features of the blood film, and a small number of irreversibly sickled cells is often seen, particularly in the β^o type. The MCV and MCH are greatly reduced, and the reticulocyte count mildly elevated.

The hemoglobin pattern of the β^o type consists almost totally of HbS with a mild increase in HbF and HbA_2. There is no HbA. The β^+ type consists of HbS, 10–30% HbA, and a mild increase in HbF and HbA_2. The electrophoretic pattern of the β^+ type with HbS well in excess of clearly discernible HbA is characteristic and unlikely to be mistaken for any other sickle hemoglobinopathy. Cases with very small amounts of HbA may be diagnosed as homozygous sickle cell disease, the HbA being visually lost in the increased HbF. Agar gel

electrophoresis widely separates HbA and HbF, and facilitates identification of the small amount of HbA. The pattern of the β^o type closely resembles that of homozygous disease, and the electrophoretic differential diagnosis usually depends on the demonstration of an increased HbA_2 level in the former condition. Examination of other family members for evidence of the thalassemia gene should be undertaken in all putative cases of homozygous sickle cell disease to avoid diagnostic error. Globin chain synthesis studies may be helpful in doubtful cases.

SICKLE CELL HbC DISEASE

Sickle cell HbC disease results from the inheritance of the HbS gene from one parent and the HbC gene from the other. In general, although it resembles homozygous sickle cell disease clinically, it is less severe. Growth, body habitus, and sexual development is normal. Most patients have painful crises and attacks of acute febrile pulmonary disease, but they are usually well between the crises, and the disease is compatible with longevity. Eye complications are often a prominent feature. Pregnancy occurs more frequently than in homozygous diseases, but in almost as hazardous for mother and child as in the latter condition. Thromboembolic episodes and hematuria are particularly common. Jaundice is unusual, although about 60% of adult patients have splenomegaly.

The patients are usually only mildly anemic or may have a normal hemoglobin level. Numerous target cells are seen on the blood film, but irreversibly sickled cells are often not present. MCV and MCH are usually mildly reduced, and the reticulocyte count is mildly elevated.

SICKLE CELL HbD DISEASE

This disease is rare; it results from the inheritance of the HbS gene from one parent and the HbD gene from the other. It occurs mainly in Blacks, but Caucasians are occasionally affected. Clinically, it resembles homozygous sickle cell disease, but is less severe and the patients are mildly anemic. Most have a normal habitus and lead a relatively normal life with only very occasional painful crises. Numerous target cells are seen on the blood film. The electrophoretic pattern may be confused with that of homozygous sickle cell disease.

OTHER HEMOGLOBINOPATHIES

HEMOGLOBIN C HEMOGLOBINOPATHIES

HbC appears to have originated in West Africa where it affects 20% of the population in some areas. The prevalence rate in the United States among Blacks is 2–3%. It has occasionally been observed in Italians. HbC arises from the substitution of lysine for glutamic acid in the sixth position of the β chain. It is less soluble than HbA, and if present in sufficient amounts tends to form crystals within the red cell. The intracellular crystals may be seen in a wet preparation or after incubation of blood in three percent sodium chloride solution at 37°C. Crystal formation occurs under these conditions in the red cells of patients with HbC disease and sickle cell HbC disease, but is not observed in HbC trait.

HbC is a slow-moving hemoglobin on cellulose acetate electrophoresis, migrating in the same position as HbE and HbA_2.

HbC trait (heterozygous state) is generally asymptomatic; in the blood film the presence of numerous target cells is characteristic, but in occasional cases they may not be a marked feature. Red cell lifespan is normal.

HbC disease (homozygous state) is usually a benign illness characterized by compensated hemolysis with a normal hemoglobin level or a mild to moderate anemia. It may be diagnosed as a result of a screening blood examination, but some patients have recurrent arthralgias or mild abdominal pain. Patients are of normal habitus, but splenomegaly is almost always present and there may be mild jaundice. Target cells are prominent in the blood film, ranging from 30% to almost 100%. The MCV and MCH may be mildly reduced, but are often normal. Occasional microspherocytes and normoblasts are usually present, and the reticulocyte count is often mildly elevated.

Sickle cell HbC is not uncommon, and has been mentioned earlier.

HbC β-thalassemia has been described in American Blacks and more recently in Italians, Africans and Turkish people. In Blacks, it is usually asymptomatic and splenomegaly is uncommon. In other racial groups, it is more severe with a clinical picture of thalassemia intermedia and frequent splenomegaly.

HEMOGLOBIN E HEMOGLOBINOPATHIES

HbE is found predominantly in South-East Asia, India, Burma and Sri Lanka and amongst immigrant Indo-Chinese populations in Western countries. Approximately 13% of the population of Thailand, Cambodia and Laos is affected. HbE arises from the substitution of lysine for glutamic acid in the 26th position of the β chain. On cellulose acetate electrophoresis, HbE is slow moving and migrates in the same position as HbC and HbA_2. Agar gel electrophoresis permits differentiation, as HbE does not separate from HbA on this medium.

HbE trait (heterozygous state) is asymptomatic, and the hemoglobin level is normal. The red cell count may be elevated. The red cells are microcytic with a mildly reduced MCV and MCH.

HbE disease (homozygous state) may also be asymptomatic in some patients. It is characterized by compensated hemolysis with a normal hemoglobin level or a mild microcytic anemia. There may be mild jaundice, and the liver and spleen are usually not enlarged. In the blood film, there is marked hypochromia, usually with many target cells. The MCV and MCH are reduced.

HbE α-thalassemia is relatively common in Thailand. It is a more severe condition than HbE disease. The clinical and hematological features resemble those of β-thalassemia major in most cases. Occasional patients are less severely affected. Death from infection in childhood is frequent, but some patients live until adult life.

HbE β-thalassemia is also common in Thailand. Several disorders of variable severity involving interactions between the α-thalassemia 1 and α-thalassemia 2 genes and HbE have been described.

HEMOGLOBIN D HEMOGLOBINOPATHIES

HbD occurs mainly in North-West India, Pakistan, and Iran, although it was first described in the United States. About three percent of Sikhs living in the Punjab are affected. It is also found sporadically in Blacks and Europeans, the latter usually coming from countries that have had close associations with India in the past. The original HbD was called HbD Los Angeles, and it was later shown to be identical to HbD-Punjab found in India and Pakistan. A number of other rare abnormal hemoglobins, both α chain and β chain and are also referred to as HbD. HbD-Punjab arises from the substitution of glutamine for glutamic acid in the 121st position of the β chain.

The electrophoretic mobility of HbD on cellulose acetate is identical to that of HbS on Agar gel electrophoresis, HbD migrates with HbA, whereas HbS separates from HbA. HbD does not sickle.

UNSTABLE HEMOGLOBIN: HEMOGLOBINOPATHIES

The unstable hemoglobins are hemoglobin variants that undergo denaturation and precipitate in the red cell as Heinz bodies. Their presence results in a rare form of congenital non-spherocytic hemolytic anemia.

The stability of the hemoglobin molecule depends on the maintenance of the normal configuration and internal contacts of the globin chains and on constant structural relationship between the heme groups and the surrounding heme pocket. The heme is held in position in the heme pocket by bonds between it and the amino acids of the surrounding chain. In the unstable hemoglobins, the firm binding of the heme group within the molecule is distributed by replacement or deletion of one or more critical amino acids. Loss of the integrity of the heme pocket leads to the formation of methemoglobin and precipitation of the globin. The precipitated globin adheres to the inner surface of the red cell membrane as Heinz bodies.

The spleen pits the Heinz bodies from the affected red cells as they circulate through it, and the resulting membrane damage leads to premature cell destruction. The structural abnormality of the heme pocket that causes hemoglobin instability leads in some cases to altered oxygen affinity of the molecule, which may influence the severity of symptoms.

Over 100 unstable hemoglobins have been described. They include Hb-Koln, Hb-Zurich, Hb-Hammersmith, and Hb-Sydney. Most arise from β-chain substitutions, and affected patients are heterozygous for the unstable hemoglobin and HbA. The condition is not limited to any particular racial group. Autosomal dominant inheritance has been noted in most families studied. In some cases, there is no family history.

LABORATORY DIAGNOSIS

Demonstration of Heinz Bodies

Preformed Heinz bodies usually cannot be demonstrated by supravital staining in the red cells of patients with an unstable hemoglobin unless a splenectomy has been performed or hemolysis exacerbated by the administration of oxidant drugs. Sterile incubation of affected red cells at 37°C for 24 hours usually leads to their formation. Following splenectomy, Heinz bodies are present in at least 50% of red cells.

Heat Instability Test

Hemoglobin instability may be directly demonstrated by this useful test which should always be used in the investigation of a patient with congenital non-spherocytic hemolytic anemia. A fresh hemolysate is incubated with phosphate or *tris* buffer at 50°C for up to two hours. A precipitate rapidly forms if an unstable hemoglobin is present.

Isopropanol Precipitation Test

This test is slightly more convenient than the heat instability test. A fresh hemolysate is incubated with an isopropanol *tris* buffer at 37°C for up to 60 minutes. A precipitate rapidly forms if an unstable hemoglobin is present.

Hemoglobin Electrophoresis

Demonstration of the unstable hemoglobin by electrophoresis is often less satisfactory than with stable hemoglobin variants. An abnormal band is present on cellulose acetate electrophoresis at pH 8.6 in about 75% of the patients, but the band is often indistinct or weak, especially if the hemolysate is not fresh. The electrophoretic mobility of Hb-Koln, the most common of the unstable hemoglobins, is similar to that of HbS. The proportion of unstable hemoglobin is highly variable. In most cases it is about 30%, but it may be as low as 2% or as high as 40%. HbA_2 and HbF are elevated in occasional patients.

Clinical and Laboratory Findings

The clinical severity of the unstable hemoglobin hemolytic anemias varies greatly. The anemia may be so mild that the patient is unaware of any abnormality, and in an occasional case there may be no anemia, the only sign of the disease being an elevated reticulocyte count. Alternatively, the anemia may be severe and evident in the first year of life. Infection or the administration of oxidant drugs may cause an exacerbation of hemolysis.

Hb-Koln is the most commonly encountered unstable hemoglobin. It causes a well-compensated chronic hemolytic anemia of mild to moderate severity with intermittent mild jaundice and splenomegaly. Cholelithiasis may occur, and the passage of dark urine is a feature in some patients.

The blood film shows only minor abnormalities, with minimal anisocytosis and poikilocytosis, variable hypochromia, punctate basophilia, and polychromasia. The reticulocyte count is always elevated, and the MCH is usually reduced. Red cell changes are more marked in splenectomized patients. Occasional patients have a mild thrombocytopenia. The mean red cell life span is 20–30 days. Exacerbation of hemolysis by drugs has not been noted with Hb-Koln.

The approach to treatment varies with the severity of the hemolysis, but many patients manage satisfactorily with a little medical attention and attain a normal life span. Blood transfusion is necessary for a sudden fall in hemoglobin level, and splenectomy has been useful in selected patients. Prompt therapy of infections and avoidance of oxidant drugs are important measures.

HEMOGLOBINOPATHIES ASSOCIATED WITH POLYCYTHEMIA

The movement of oxygen into and out of the hemoglobin molecule depends on changes in spatial relations between the α and β globin chains. Amino acid substitutions that result in alterations of oxygen affinity are generally situated at critical areas of chain contact and interfere with the normal process of chain rearrangement associated with oxygenation and deoxygenation.

When the amino acid substitution impairs ability to release oxygen at the tissue level, the abnormal hemoglobin is referred to as a high oxygen-affinity hemoglobin. The resulting anoxia stimulates compensatory erythropoietin production and hence erythrocytosis. The *in vitro* counterpart of the *in vivo* abnormality is the abnormal oxygen dissociation curve, which is 'shifted to the left' when whole blood from an affected patient is examined.

The shift reflects the increased oxygen affinity, the fall in oxygen saturation for any given drop in partial pressure of oxygen being less than that found in normal adult blood.

At least 25 high oxygen-affinity hemoglobins with erythrocytosis have been described, the majority being β-chain substitutions, inherited as autosomal dominants. Affected patients are heterozygotes.

LABORATORY DIAGNOSIS

Hemoglobin Electrophoresis

An abnormal electrophoretic band is demonstrable on cellulose acetate at pH 8.6 in about half of the patients. Separation of the abnormal hemoglobin on Agar gel electrophoresis at pH 6.0 may be successful in some instances even in the absence of a cellulose acetate band, but in a significant number of cases no band can be demonstrated by conventional techniques. The abnormal hemoglobin constitutes 20–50% of the total hemoglobin.

Oxygen Dissociation Studies

These are essential for definitive diagnosis, but are often not available outside specialist referral centers. Blood samples despatched by airmail may be satisfactory for analysis. The normal P50 value for whole blood is 25 to 30 mm Hg. In patients with high affinity hemoglobins, levels range from 12 to 20 mmHg.

Clinical and Laboratory Findings

Affected individuals have a hemoglobin level, packed cell volume, and red cell count at or above the upper limits of normal. White cell and platelet count are normal, and there is no splenomegaly. The patients are usually plethoric, but have no symptoms. Investigation of the plethora or a chance screening hemoglobin estimation may lead to detection. Blood gas analysis is normal. The absence of leukocytosis and thrombocytosis clearly distinguishes the disorder from polycythemia rubra vera. More pertinent is the distinction from other forms of secondary erythocytosis, familial or otherwise. If no obvious cause for the increased hemoglobin level is apparent, and especially if the subject is young and a family history with a dominant pattern of inheritance is obtained, the presence of an abnormal hemoglobin should be suspected. If an abnormal electrophoretic band cannot be demonstrated on cellulose acetate or agar gel electrophoresis, oxygen dissociation studies are necessary. Family members should be examined if available. The erythrocytosis of the high oxygen-affinity hemoglobins appear to be benign since the clinical syndrome has been found in apparently well subjects beyond middle age. Treatment is usually not necessary, but an occasional patient has benefited from phlebotomy. The high oxygen-affinity hemoglobins include Hb-Chesapeake, Hb-J Capetown, Hb-Kempsey, and Hb-Ypsilanti.

THALASSEMIAS (REDUCED SYNTHESIS RATE)

These are a heterogeneous group of disorders with a genetically determined reduction in the rate of synthesis of one or more types of normal hemoglobin polypeptide chain. This results in a decrease in the amount of the hemoglobin involving the affected chain. In some forms of thalassemia, the genetic mutation results in the synthesis of a structurally abnormal hemoglobin which is produced at a reduced rate.

Thalassemia was originally described in Italians, Greeks, Spaniards and other people of Mediterranean origin. However, it is now realized that it is a common disorder with a widespread geographical distribution. It also occurs in the Middle-East, India, South-East Asia, and in Blacks. Persons of Mediterranean origin in non-European countries may be affected. No population group is completely free from the condition, and it is now occasionally identified in persons of Northern European origin.

CLASSIFICATION

The α and β chains of hemoglobin are synthesized independently under separate genetic control and, in the normal state, synthesis of the two chains is balanced. There are two main groups of thalassemia, one affecting the synthesis of α chains, and the other affecting the synthesis of β chains; these are called α-thalassemia and β-thalassemia respectively.

In β-thalassemia, the inadequate production of β chains leads to a reduction in the amount of HbA in the red cell, and a microcytic hypochromic anemia results. The total hemoglobin is maintained in part by the production of γ and δ chains, and thus increased HbF or HbA_2 is usually found. The lack of β chains leads to accumulation of free uncombined α chains within the developing red cells. These chains aggregate and interfere with erythroid cell maturation and function, resulting in premature destruction of the cells in the marrow and consequent ineffective erythropoiesis

In α-thalassemia, the levels of HbA, HbF and HbA_2 are equally depressed since they all have β chains; there is usually a microcytic hypochromic anemia. In the absence of sufficient α chains, excess β chains or γ chains aggregate to form HbH (β_4) or Hb-Barts (γ_4). The inheritance of thalassemia is co-dominant, and follows classic mendelian principles α and β-thalassemias exist in both the homozygous and heterozygous states, and the genes for thalassemia may interact with those of the hemoglobin structural variants.

LABORATORY DIAGNOSIS

The diagnosis of thalassemia is initially suggested by the clinical findings, the patient's ethnic origin and family history, and the results of routine hematological tests. In the diagnosis of β-thalassemia, HbA_2 is quantitated by hemoglobin electrophoresis or microcolumn chromatography, and HbF by the alkali denaturation test. In α-thalassemia, red cell HbH inclusions are demonstrated by specific stains, and hemoglobin electrophoresis is used to detect HbH and Hb-Barts. A definitive diagnosis may be made at this point in most cases. Globin chain synthesis rate and gene analysis studies, which require the assistance of a reference laboratory, are sometimes useful in difficult cases.

Routine Hematological Tests

The hemoglobin level, packed cell volume, red cell count, red cell indices, reticulocyte count, and examination of a stained blood film are essential initial tests. The MCV, which is measured electronically in many laboratories, is particularly useful. An MCV result of <80 fL alerts the clinician to the diagnosis of α- or β-thalassemia, although iron deficiency and a number of chronic illnesses frequently seen in hospital practice also cause low levels. Serum iron, total iron-binding capacity, ferritin, bilirubin, and other biochemical parameters of hemolysis should be measured.

Demonstration of HbH Inclusions

When red cells containing HbH are incubated with a solution of a redox dye (e.g. brilliant cresyl blue), HbH, which is relatively unstable, precipitates and the red cells are pitted by numerous inclusions, an appearance likened to the surface of a golf ball. The inclusions must be distinguished from the reticulin of reticulocytes and from preformed Heinz body-like inclusions, which are numerous after splenectomy in patients with HbH disease.

Hemoglobin Electrophoresis

Precise measurement of the HbA_2 level is required for the diagnosis of several types of β-thalassemia. Hemoglobin electrophoresis on cellulose acetate at pH 8.6 is widely used, the HbA_2 band being eluted from the strip and measured spectrophotometrically. A number of accurate microcolumn chromatographic methods are also available, some in prepackaged kit form. They are quicker and more convenient to perform than electrophoretic methods and have replaced the latter in many laboratories. If present in sufficient amounts, HbH and Hb-Barts may be demonstrated by electrophoresis on cellulose acetate and starch gel at pH 8.6. They are referred to as "fast" hemoglobins because they migrate towards the anode.

Globin Chain Synthesis Rate Studies

Studies of incorporation of radioactive amino acids into hemoglobin chains in immature red cells as a measure of relative rates of α- and β-chain syntheses have demonstrated unbalanced synthesis in α- and β-thalassemias. These investigations are hardly ever needed in routine practice but are important tools in antenatal diagnosis.

CLASSIFICATION OF THALASSEMIAS

Clinical

Thalassemia major:

- Transfusion dependent
  - Homozygous β°-thalassemia
- Homozygous β^{+}-thalassemia (some types)

Thalassemia intermedia:

- Mild forms of compound $\beta^{+}\alpha^{+}\beta^{\circ}/\beta^{+}$-thalassemia
- Hemoglobin lepore syndromes
- Homozygous δβ-thalassemia and hereditary persistence of fetal hemoglobin
- Combination of α- and β^{+}-thalassemia
- Heterozygous β-thalassemia with triplicated β genes
- Dominant β-thalassemia and β chain
- Heterozygosity for variants, e.g. HbE β-thalassemia
- Hemoglobin H disease.

Thalassemia minor

- β°-thalassemia trait
- δβ-thalassemia trait
- Hereditary persistence of fetal hemoglobin
- β^{+}-thalassemia trait
- α_1 (α°)-thalassemia trait
- α_2 (α^{+})-thalassemia trait

In β-Thalassemia

There is reduced β-chain production, therefore, HbA produced is less and the anemia produced is microcytic, hypochromic anemia. Total Hb is maintained by increase in γ and δ chains so HbA_2 and F increase. Because of lack of β chains, α chains accumulate in cells forming aggregates—ineffective erythropoiesis (Table 9.4).

Table 9.4: Classification of β-thalassemia		
Type	*Heterozygous*	*Homozygous*
β°	Thalassemia minor HbA_2 >3.5%	Thalassemia major HbF 98%, $HlbA_2$ 2%, no HbA
β^+	Thalassemia minor HbA_2 >3.5%	Thalassemia major HbF 70–80%, HbA 10–20% HbA_2 variable
δβ hereditary persistence of fetal hemoglobin	Thalassemia minor HbF >5–20% HbA_2 normal or low	Thalassemia intermedia, HbF 100%
Hb-Lepore	Thalassemia minor HbA >80–90% Hb lepore l0%	Thalassemia major or intermedia HbF 80%, HbA_2 reduced, Hb-Lepore 10–20%, HbA, HbA_2 absent

In α-Thalassemia

Levels of Hb, A, A_2 and F are equally depressed and there is microcytic hypochromic anemia. In the absence of α chains, β- or γ chains are produced more and form HbH (β_4) or Hb-Barts (γ_4). α- and β-thalassemias exist in both homozygous and heterozygous states and may interact with other hemoglobins (Table 9.5).

Table 9.5: Classification of α-thalassemia			
Designation	*Haplotype*	*Heterozygous*	*Homozygous*
α°-thalassemia	–/	α°-thalassemia, MCH, MCV low	Hydrops fetalis
Dysfunctional α–thalassemia	– α°	α°-thalassemia	Hydrops MCH, MCV low fetalis
α^+-thalassemia	– α	α^+-thalassemia, minimal, If any, hematological abnormality	As heterozygous α°-thalassemia
Nondeletion α-thalassemia	αα	Variable	HbH disease in some cases
Hb-constant spring (CS)		0.5–1% HbCS	More severe than heterozygous α^1-thalassemia

The combination of α^1-thalassemia (or dysfunctional α-thalassemia) and α^2-thalassemia gives rise to HbH disease.

β-THALASSEMIAS

The genetic mutation of β-thalassemia leads to a decreased rate of β-chain synthesis and consequently a reduction in the amount of normal HbA in the red cell. A microcytic hypo- chromic anemia results. On the basis of the extent of reduction of β-chain synthesis, two main types of β-thalassemia are recognized. β^+-thalassemia is characterized by incomplete suppression and β^o-thalassemia by complete absence of chain synthesis. Both β^o and β^+ types occur throughout the Mediterranean region. β^o-thalassemia predominates in South-East Asia, and β^+ is the usual type in Blacks. Although the two types cannot always be distinguished on clinical grounds in individual patients, the variable severity of β-thalassemia in some population groups is ascribed in part to the existence of these two thalassemia genes.

Recent gene analysis studies have established that β-thalassemia may arise from any of between 40 and 50 different mutations of the β globin gene. The great majority are single base changes, and gene deletion is only rarely a cause. Most of the mutations affect the RNA splicing mechanism or block translation of mRNA into protein.

At the clinical level, β-thalassemia occurs classically in two forms. β-thalassemia major, or Cooley's anemia, is usually a severe illness characterized by major or total suppression of chain synthesis and is the homozygous form of the disease. β-thalassemia minor, or trait, is a mild and sometimes asymptomatic condition and represents the heterozygous form. Suppression of β-chain synthesis is much less severe.

Some patients do not fit easily into these two clear-cut clinical categories. Patients may be clinically classified as thalassemia intermedia if the severity of their disease lies between that of the major and minor forms. Thalassemia intermedia encompasses a range of interactions between many different thalassemia genes which result in milder defects of β-chain synthesis and globin-chain imbalance than occur in classical β-thalassemia major.

A classification of the commonly occurring types of β-thalassemia is outlined below.

The β-thalassemias and related syndromes: Electrophoretic phenotypes.

	Hemoglobin (%)		
Disorder	***A***	***A_2***	***F***
β-thalassemia minor	90–95	3.5–7.0	1–5
δβ-thalassemia minor	80–95	1.0–3.5	5–20
β-thalassemia major			
$\beta\text{-thal}^{+}$	10–20	1.5–4.0	70–80
$\beta\text{-thal}^{0}$	0	1.5–4.0	98
HPFH (Black heterozygote)	60–85	1.0–2.0	15–35

HPFH = Hereditary persistence of fetal hemoglobin.

β-THALASSEMIA MINOR (TRAIT)

This condition is the heterozygous state for the β-thalassemia gene. It is characterized by a moderate reduction in β-chain synthesis as directed by a β-thalassemia gene inherited from one parent. The disorder is relatively common, e.g. it has been found in 8% of Greeks and 5% of Italians. Clinically, it is usually a very mild disorder with little or no anemia, no symptoms, and a normal life expectancy. The spleen may be palpable. The condition is commonly not diagnosed until adolescence or adult life, and may be detected in a routine hematological screening examination. It is often first diagnosed in pregnancy.

The hemoglobin level is usually normal or mildly reduced, but rarely less than 10 g/dL. The red cell count is often normal despite mild anemia, and may be increased. The MCV and MCH are reduced, with values of 63–77 fL and 18–25 pg respectively. The MCHC is usually marginally reduced or normal. In occasional patients with concurrent α-thalassemia, the red cell indices are normal. Examination of the blood film shows mild red cell anisocytosis, microcytosis, and hypochromia with variable numbers of target and stippled cells. The osmotic fragility test shows an increased resistance to hemolysis even when anemia is absent. The serum bilirubin is normal or slightly raised.

In the great majority of cases, HbA_2 is increased. A small increase in HbF occurs in about 50%; thus the absence of any increase in HbF does not exclude the diagnosis.

The main problem in diagnosis is the differentiation of β-thalassemia minor from iron deficiency in a person of Mediterranean origin. Clinical features are often not helpful. In the usual case of thalassemia minor, red cell microcytosis, hypochromia, and reduction in MCV are relatively marked considering the mild or absent anemia. This is not the case in iron deficiency in which there is closer correlation between the morphological abnormalities

and the degree of anemia. Red cell anisocytosis, as measured by the cell sizing facility on some modern electronic cell counters, is often more marked in iron deficiency.

Estimation of the serum iron, ferritin and transferrin, and of the red cell HbA_2 and HbF, usually provides a definitive diagnosis. When iron deficiency develops in a patient with thalassemia minor, the elevated HbA_2 usually falls to normal but returns to a supranormal level when iron stores are replenished. If the HbA_2 and the HbF levels are normal, and the patient is not iron deficient, the possibility of α-thalassemia should be considered and the test for HbH inclusions performed.

Treatment is generally not required in mild cases, and the benign nature of the disorder should be emphasized to the patient. Careful surveillance of the hemoglobin level during pregnancy is advisable, and prophylatic folic acid should be given. Although the patient with thalassemia minor may become iron deficient, it is more usual for patients to receive oral or parenteral iron therapy for many years on the mistaken assumption that all hypochromic anemias are due to iron deficiency. Thus, it is important to inform the patient of the diagnosis and the possible harmful effects of over-enthusiastic iron therapy.

Heterozygous state, clinically mild with little or no anemia. Patient lives a normal life, splenomegaly may or may not be present.
Detected—on routine, examinations, often in pregnancy. Very rarely one may present with icterus, splenomegaly, leg ulcers or radiographic changes of long bones.
Because hematologic alterations resemble iron deficiency anemia these women are treated with iron supplementation (wrongly). These patients have increased iron absorption and may land up with hemosiderosis ultimately.

Blood Picture

Hemoglobin is normal, MCV and MCH are reduced. MCH is normal peripheral smear shows

- Microcytic hypochronic cells, target cells
- Basophilic stippling, reticulocytosis (up to 6%)
- RBC count is raised, no nucleated red cells seen
- Osmotic fragility is increased
- Evidence of hemolysis
- Serum bilirubin may be slightly elevated
- Erythrocyte free protoporphyrin is usually normal (in contrast to elevated levels in iron deficiency anemia)
- Bone marrow is normal except for mild erythroid hyperplasia
- RBC life is only slightly shortened with modest ineffective erythropoiesis.
- Electrophoresis; HbA_2 increased, slight increase in HbF.

Differential diagnosis: Deficiency anemia—in this there is no jaundice and splenomegaly is absent. In thalassemia, the number of target cells, degree of punctate basophilia and raised osmotic fragility are greater

Estimation of Fe, TIBC, HbA_2 and HbF provide a definitive diagnosis.

β-THALASSEMIA MAJOR

Beta-thalassemia major is the homozygous state for either the β^o or β^+-thalassemia gene or, less commonly, the heterozygous state for the two genes. In its usual form in Italian or Greek patients, it is severe disease which often results in death during childhood unless frequent blood transfusions are given. About one in every ten patients has a mild form which is compatible with survival into adult life with only occasional transfusions. This form of the disorder may be referred to as thalassemia intermedia, although the hemoglobin constitution is usually identical to that seen in classical thalassemia major. Extreme

examples of the mild form, which sometimes occur in American Blacks, are completely symptomless or have only a mild anemia. In some cases, the clinical heterogeneity appears to be due to the interaction of other genetic (for example, α-thalassemia or hereditary persistence of fetal hemoglobin) or environmental factors with the β-thalassemia gene.

Clinical

- Neonate with β-thalassemia major is not anemic.
- Insidious onset, pallor appears in first year of life
- Abdominal girth expands
- Patients fail to thrive
- In the absence of transfusion therapy, hemoglobin falls to 3–5 g%
- Stature is shortened, head is large and abdomen protrudes
- Prominence of the cheek bones tends to obscure base of the nose and expose the upper teeth
- Thickening of the cranial bone produces frontal bossing
- Mild jaundice may be present together with enlargement of liver, spleen, and heart.

Skeletal Changes

- These changes result from marrow expansion and cortical thinning.
- The earliest changes appear in hands and feet, the metacarpals, metatarsals and phalanges become rectangular or frankly convex-shaped.
- Trabeculation of marrow spaces gives the bones a mosaic pattern.
- Skull and facial bones show striking changes: The dipole of the skull is thickened several times the normal, the outer and inner tables are thin, and perpendicular striations appear between the tables—hair-on-end appearance.
- Pneumatization of sinuses is delayed.
- In the long bones widening of medullary portions and thinning of the compact bone of the cortex occur, predisposing to pathologic fractures. Compression fractures of vertebrae may occur.
- The ribs are broad especially at the vertebral junction.

Growth and Sexual Development

- Growth retardation in early childhood occurs because of anemia.
- Puberty is delayed and is often incomplete
- Secondary sex characteristics are conspicuously absent or subtle
- Girls are amenorrheic or oligomenorrheic and sterile
- Boys may have active spermatogenesis but lack libido.
- There is failure either of hypothalamic maturation or pituitary function
- Reserves of adrenal cortical hormones, parathormone, thyroxine and glucagon appear to be diminished.

Cardiopulmonary Complications

- After the first decade many patients experience sterile pericarditis (it is self-limited though).
- More important is myocardial hemosiderosis, the leading cause of death in transfused patients. Once developed, survival is usually for months only.
- Pulmonary function tests are indicative of small airway obstruction and hyperinflation.

Hepatobiliary Disease

- Hepatomegaly (early) is due to myeloid metaplasia but later results from cirrhosis with regenerative nodules
- Microscopically it resembles idiopathic hemochromatosis
- Pigmentary gallstones are not uncommon in patients after 4 years of age
- Hemosiderosis
- There is increased body iron because of:
  1. Repeated transfusions
  2. Raised intestinal absorption that occurs because of ineffective erythropoiesis and chronic anemia
- Hypersplenism—causes pancytopenia

Blood Picture (Fig. 9.5) (Resembles Iron Deficiency Anemia)

- Marked microcytosis with hypochromia (MCV 48–72 fl and MCHC 23–32 g%)
- Moderate degree of anisopoikilocytosis, tear drop cells
- Target cells are prominent
- All MCV, MCH, MCHC are diminished
- Normoblasts (intermediate/late) in peripheral smear
- Granular inclusions in cytoplasm represent α-chain aggregates which can be shown by methyl violet (following splenectomy)
- Polychromasia and moderate degree of punctate basophilia
- Reticulocytosis (10%)
- Leukocytosis (5,000–40,000), shift to the left

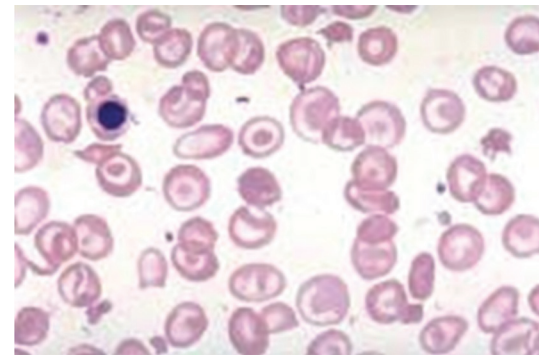


Fig. 9.5: β-Thalassemia, peripheral smear

- Platelet count normal but decreases with splenomegaly
- Osmotic fragility decreased, the curve has a tall because of a few very fragile cells
- Evidences of intravascular hemolysis.

Bone Marrow

- Erythroid hyperplasia
- Micronormoblastic reaction
- Increase in early and intermediate normoblasts
- Methyl violet positive inclusion bodies and PAS positive glycogen in normoblasts
- Sideroblastosis, often ring sideroblasts
- Macrophages similar to Gaucher cells may be seen.

Hb Pattern

- HbF (10–98%)
- HbA (very little or absent)
- HbA_2 (variable).

Laboratory Diagnosis

Clinical features
Patient's ethnic origin
Family history
Routine blood tests
} Suggests diagnosis

β-Thalassemia

- HbA_2 quantification by electrophoresis
- HbF by alkali denaturation and acid elution test, found to be raised

Globin chain reduction studies

- In difficult cases, incorporation of radioactive amino acids in Hb chains.

Routine blood tests

- MCV <76 fL

(Differential diagnosis: Iron deficiency anemia and anemia of chronic illness).

Pathophysiology of Anemia

Anemia occurs because of:

- Intramedullary red cell death (because of precipitation of globulins)
- Reduced life span
- Impaired heme synthesis
- Peripheral hemodilution
- Red blood cells with inclusions are pitted in spleen

There is dual red cell population—one with a short life span and the other with longer lifespan (these have greater amount of HbF).

Prognosis

Life expectancy with α-thalassemia major is 15–25 years (untreated will die within 5 years)

If regularly transfused, patients die in 2nd or 3rd decade because of intractable congestive heart failure, cirrhosis, diabetes mellitus—all because of hemochromatosis.

Splenectomy is indicated when:

- Large spleen causes discomfort
- Transfusions are needed at very short intervals.

Therapeutics

By and large most patients with β-thalassemia major are severely anemic, and many of the distressing symptoms of the conditions are directly related to the anemia. The compensatory mechanisms utilised by the body to improve the production of viable red cells may also cause symptoms, and in some patients are more troublesome than the anemia itself. The expanding hyperplastic bone marrow leads to gross skeletal changes, particularly affecting the face; masses of extramedullary erythroid tissue compress vital structures; and increased iron absorption cause deposition of iron in parenchymal tissues. Thus, blood transfusion which alleviates the anemia and suppresses the compensatory mechanisms is the basis of therapy.

The original aim of transfusion therapy was to maintain the hemoglobin at the lowest safe level. This usually involved blood transfusion at 5–10 weekly intervals. More frequent transfusions were considered unwise because of the iron content of the transfused blood (each unit of blood contains 250 mg iron) and the likelihood of accelerated development of hemosiderosis and organ failure.

Of late, it has been understood that an intensive transfusion policy offers considerable advantages for the patient, not withstanding its additional contribution to body iron accumulation. Current hypertransfusion regimens are commenced in the first or second year of life and aim at maintaining a mean hemoglobin level of at least 10 g/dl by transfusion of concentrated red cells at 4–6 weekly intervals. Children treated in this way remain well with a normal pattern of growth and development until the early teens, when the effects of iron overload gradually appear. It is important to establish the patient's steady-state hemoglobin level before embarking on an intensive transfusion regimen. The patient should be phenotyped, and group-specific red cells (preferably rendered leukocyte-poor) should be used, if possible. To ensure prolonged *in vivo* survival, fresh blood is desirable, and a technique involving the transfusion of young red cells ('neocytes') obtained from donors by means of a cell separator is used in some clinics.

In order to prevent the inevitable accumulation of iron, the iron-chelating agent desferrioxamine (DF) is used. DF is able to remove substantial amounts of storage iron via urine and stool, and, if commenced early in life, preferably at the same time as the regular transfusion regimen, will prevent (or at least delay) the occurrence of iron overload. In older patients with established iron overload, DF curtails further accumulation of hepatic iron and progression of fibrosis. In most cases, hepatic iron content decreases, liver function tests improve, and the level of serum ferritin falls. The drug is administered subcutaneously using a small portable infusion pump; the needle is inserted into the anterior abdominal wall and the dose infused over a 12-hour period daily for 5 days per week. Preliminary studies to establish a dose response curve are advisable to arrive at an optimal dosage regimen. The average daily dose for children is 50–60 mg/kg/day, and for adults approximately 2 g per day. The addition of vitamin C, 200 mg orally on each day of chelation, enhances iron excretion. In spite of its high cost, complexity of administration and potential for visual and auditory neurotoxicity DF is now used widely in the treatment of children with thalassemia major.

Huge splenomegaly is less of a problem since the advent of hypertransfusion regimens. Splenectomy has a definite place in the management of two groups of patients: in children with massive splenomegaly causing severe discomfort and symptoms due to pressure, and in patients with progressively increasing transfusion requirements. In hypertransfused patients, there is often a progressive shortening of the interval between transfusions, until ultimately transfusion is required very frequently. In more cases, immune alloantibodies cannot be demonstrated, and the increased destruction appears to be due, at least in part, to a 'hypersplenic' mechanism, since splenectomy in such patients usually causes a significant fall in transfusion requirements. Red cell survival and sequestration studies using ^{51}Cr-labelled red cells are not helpful in assessing the likelihood of response to splenectomy.

Surgical removal of spleen should not be performed, if at all possible, until after the age of five or six years. There is a major risk of life-threatening infection in children who are splenectomized before this age. The reasons for the increased susceptibility to infection following removal of the spleen are not known with certainty, but are probably similar to those in sickle cell disease. *Pneumococcus, Meningococcus* and *Haemophilus influenzae* are usually the organisms involved. Immunization with polyvalent pneumococcal vaccine is essential, and most authorities recommend prophylactic oral penicillin.

Immediate and aggressive antibiotic therapy should be commenced at the onset of the infection; provision of parenteral antibiotics to the parents of affected children for administration at the first sign of fever may be worthwhile.

Management of the various syndromes of organ failure that characterize the later course of the thalassemic patient is often difficult. Iron deposition in cardiac muscle causes arrhythmias and chronic congestive cardiac failure which may be refractory to conventional therapy. The myocardium is very sensitive to digitalis, and consultation with an experienced cardiologist is advisable. Episodes of pericarditis are managed by bed rest, treatment of heart failure, and antibiotics if necessary. Spinal cord compression from masses of hyperactive erythroid tissue may require myelography and radiation therapy or laminectomy. Occasionally, patients develop mild megaloblastic bone marrow changes, and a maintenance dose of folic acid may be warranted.

The place of bone marrow transplantation in the management of β-thalassemia major is currently under investigation. Some researchers have reported promising early results, but long-term data are not available and the procedure is associated with considerable morbidity from graft versus host disease and infection.

Ideally, children with thalassemia should be treated in centers set-up for their management. This permits establishment of the close relationship between patient, parents, and clinician which is essential for successful care of a serious chronic illness.

Prevention

Drives to screen and detect asymptomatic, previously unknown, thalassemic heterozygotes and to provide information and genetic counselling have been established in several nations in which the thalassemia gene is prevalent throughout the whole population or in sectional ethnic groups.

The measurement of red cell MCV using an electronic cell counter has usually been used as the basic screening test and have proven to be of great help.

Antenatal diagnosis and selective termination in pregnancies at risk for homozygous β-thalassemia, Hb-Barts hydrops fetalis and homozygous sickle cell disease are now available in a limited number of specialized centers. Two methods are used:

Measurement of Globin Chain Synthesis

A small sample of fetal blood is obtained at the 18th–20th week of gestation, either by placental needling under ultrasound guidance or by fetoscopic umbilical vein aspiration. Globin chain synthesis rates in fetal reticulocytes are measured and the β : γ synthesis ratio is used to identify fetuses that are heterozygous or homozygous for the gene of interest. In a recent international survey conducted fetal mortality associated with the procedure was 5.4% overall, and there was a 0.8% diagnostic error rate.

Analysis of Fetal DNA

Amniotic fluid is obtained by transabdominal amniocentesis during the 15th–18th week of gestation. Recently, first trimester diagnosis has been possible in some cases by using trophoblastic tissue obtained by transcervical aspiration (CVS or chorionic villous Sampling), and it is expected that this method will be increasingly used in the future. Medical termination of pregnancy is easier in the first trimester if required.

Analysis of fetal DNA is performed by gene mapping techniques after extraction from fetal fibroblasts or chorion. The DNA is cut at specific sequences into small fragments with restriction enzymes obtained from bacteria. The resulting fragments are separated according to size by agarose gel electrophoresis and transferred onto nitrocellulose paper by a process called Southern blotting. The particular gene of interest is identified by hybridization with a specific radioactive probe, followed by autoradiography. The

restriction fragments that are complementary to the probe hybridize with it and form a band on the X-ray film.

By utilizing the above technique, several approaches have been used in the prenatal diagnosis of hemoglobin disorders.

Direct identification of mutant genes: The disorders in which the basic genetic abnormality is either a substantial gene deletion or a base substitution that alters a restriction enzyme cleavage site can be identified directly by gene mapping. These include Hb-Barts hydrops fetalis in which there is deletion of the α gene, sickle hemoglobinopathies in which the base substitution alters a restriction site, and some types of β-thalassemia. The majority of β-thalassemias cannot be analyzed in this way, but is likely that a new technique using synthetically produced short DNA fragments (oligonucleotides) as probes will be increasingly used for these disorders in the future.

Restriction fragment length polymorphism (RFLP) analysis: RFLPs are normal variations (polymorphisms) in DNA structure between one individual and another that occur about once in every 100–200 nucleotide bases. They either remove a restriction enzyme cleavage site or introduce a new one, and may be analyzed by gene-mapping techniques. They are inherited in a Mendelian fashion, and can be used as genetic markers to trace through families the mutant genes to which they are linked. RFLPs are common in the region of the β globin gene complex, and the technique has been widely used in the prenatal diagnosis of thalassemia.

DELTA-BETA THALASSEMIA

This is a type of thalassemia resulting from complete absence of both β- and δ-chain synthesis, in most cases due to extensive deletion of DNA in the β globin gene complex, occurs in areas where the β-thalassemia gene is prevalent. The homozygous form of the condition is rare, and is characterized by a β-thalassemia major like illness of moderate severity and 100% HbF in the red cells. The heterozygous form is more common, and closely resembles β-thalassemia minor, both clinically and hematologically. Unlike β-thalassemia minor, however, the HbA_2 level is normal or reduced, and the only abnormality of hemoglobin constitution is an elevation of HbF which ranges from 5 to 20%. The acid elution test demonstrates a heterogeneous distribution of HbF in the red cells.

THE HEMOGLOBIN-LEPORE SYNDROMES

An occasional patient with apparent classical β-thalassemia minor or major has an abnormal hemoglobin, Hb-Lepore. Structural analysis has shown that Hb-Lepore is made up of normal α chains combined with chains that consist of parts of both δ and β chains. Three distinct types of Hb-Lepore have been described, namely Hb-Lepore Boston, Hb-Lepore Hollandia, and Hb-Lepore Baltimore. The abnormal chains are produced by δβ fusion genes which arise by unequal crossing over between parts of the δ and β globin genes during meiosis.

These Hb-Lepore syndromes are found in many population groups. They are particularly common in Southern Italy and in parts of Greece and Yugoslavia. Hb-Lepore has a similar mobility to HbS on cellulose acetate electrophoresis. It does not separate from HbA on agar gel.

The heterozygous state for Hb-Lepore is a very mild disorder resembling β-thalassemia minor. Hb-Lepore constitutes about 10% of the total hemoglobin, HbF is slightly increased, and HbA and HbA_2 make up the remainder.

The homozygous state for Hb-Lepore is rare. Clinically and hematologically it resembles β-thalassemia major. 75% of the hemoglobin is HbF, and the remainder is Hb-Lepore. There is no HbA or HbA_2.

The double heterozygous state for Hb-Lepore and β-thalassemia is more common. It also resembles β-thalassemia major. About 10% of the total hemoglobin is Hb-Lepore, and HbA may be completely absent or comprise 20–40%. The remainder of the hemoglobin is HbF and small amounts of HbA_2.

The Lepore hemoglobins have also been found in association with β-chain structural variants such as HbS and HbC.

HEREDITARY PERSISTENCE OF FETAL HEMOGLOBIN (HPFH)

A small number of apparently healthy adult subjects with normal or near normal hematological findings have a raised HbF level in most population groups. Two broad categories of this condition, which is referred to as 'hereditary persistence of fetal hemoglobin' (HPFH), have been described. In pancellular HPFH, the acid elution test shows a homogeneous distribution of HbF in the red cells, whereas in heterocellular HPFH, the HbF is heterogeneously distributed.

The common type of pancellular HPFH, the Black form, is due to deletion of part or all of the δ and β regions of the β globin gene complex, and in most respects resembles δβ-thalassemia. In the homozygous state, there is a mild thalassemia-like blood picture, and the hemoglobin consists entirely of HbF. Heterozygotes have a HbF level of about 25%, and the only hematological abnormality is a slight reduction in MCH. Patients who are heterozygous for the HPFH and HbS genes have approximately 30% HbF and 70% HbS in their red cells. In the Greek form of pancellular HPFH, as yet observed only in heterozygotes, HbF is about 15%. Heterocellular HPFH is less clearly defined, and the elevation in HbF is generally mild. In the Swiss form, HbF levels in heterozygotes are in the 2–5% range.

These HPFH syndromes may cause diagnostic difficulty as they resemble some forms of β-thalassemia. In combination with the HbS gene, the disorder may be mistaken for homozygous sickle cell disease or sickle cell β-thalassemia with complete suppression of HbA. The absence of significant hematological abnormalities in the homozygous and heterozygous states, and the mild nature of the illness when combined with HbS in addition to the homogeneous distribution of HbF in the red cells as demonstrated by the acid elution test, permits differentiation of the HPFH syndromes.

α-THALASSEMIA

These (α-thalassemias) are disorders in which there is defective synthesis of α chains with resulting depression of production of the hemoglobins that contain α chains, i.e. HbA, HbA_2 and HbF. The deficiency of α chains leads to an excess of β chains in the fetus, and of γ chains in the adult. The γ chains form the tetramer Hb-Barts (β_4), and the unstable β chains precipitate and form HbH (β_4). The presence of Hb-Barts and HbH in the red cell has serious consequences as both hemoglobins have a high oxygen affinity and thus are unable to deliver adequate oxygen to the tissues.

CLASSIFICATION (Table 9.6)

Earlier clinical and hematological studies of α-thalassemia identified four forms of the disorder. Alpha-thalassemia 2 usually showed no abnormalities on routine hematological

Table 9.6: Classification of the α-thalassemia syndromes			
Clinical	*Genetic*	*Genotype*	*Number of genes*
α-thalassemia 2	Heterozygous α^+-thalassemia	αα/–α	3
α-thalassemia 1	Homozygous α^+-thalassemia Heterozygous α°-thalassemia	–α/–α αα/– –	2 2
HbH disease	Double heterozygote 1 α^+/α°-thalassemia	–α/–	–
Hb-Barts hydrops fetalis	Homozygous α°-thalassemia	– –/– –	0

examination, and α-thalassemia 1 was a benign condition with a variable mild anemia and red cell hypochromia. The clinical picture of HbH disease was intermediate between that of β-thalassemia minor and major, and infants with Hb-Barts hydrops fetalis died *in utero* or shortly after birth.

Even though this nomenclature is still widely used, advances in methods of gene analysis have greatly increased understanding of the genetic basis of α-thalassemia, and have provided a more rational basis for classification. Normal subjects have two linked α gene loci on the short arm of chromosome 16, and thus an α gene haplotype αα/ and genotype αα/αα. The α-thalassemias are most commonly due to deletions of one or more of these genes. In most affected populations, two abnormal α gene haplotyes are found, namely α^+ (–α/) which arises from deletion of one α gene and α° (– –/) from deletion of both α genes. The majority of α-thalassemia phenotypes result from the interaction of these two haplotypes, the clinical severity depending on the number of genes deleted up to the possible four. The α^+ (–α/) haplotype usually involves the deletion of either 3.7 ($-\alpha^{3.7}$) or 4.2 ($-\alpha^{4.2}$) kilobases of DNA from the α globin gene complex. α° (– –/) haplotypes are assigned according to the geographical area in which they commonly occur, e.g. $--^{MED}/$, $--^{SEA}/$. A less common non-deletion form of α-thalassemia in which there is partial suppression of α-chain synthesis in the presence of an apparently normal complement of α globin genes ($\alpha\alpha^T$/haplotype) has also been described in some population groups.

Alpha-thalassemia is common in South-East Asia, particularly in Thailand where 20% of the population is affected. It also occurs in the Middle-East, Greece, Italy, India, Africa and the Pacific Islands. The prevalence rate among American Blacks is 25–30%. The α°(– –/) haplotype (and therefore HbH disease and Hb-Barts hydrops fetalis) is restricted to South-East Asia and, to a lesser extent, the Mediterranean countries.

ALPHA-THALASSEMIA TRAIT

Alpha-thalassemia trait: The trait is asymptomatic and is difficult to diagnose with certainty in adult life. It should be suspected in all patients of high risk ethnic origin with a refractory microcytic hypochromic blood picture once iron deficiency and β-thalassemia minor have been excluded. Two types are recognized, but in the individual case distinction is often not possible without globin chain synthesis or gene-mapping studies.

Alpha-thalassemia 2 (α^+-thalassemia): This represents the heterozygous state for α-thalassemia (–αα/–α). In the newborn period, affected infants may have 1–2% Hb-Barts which they gradually lose over the ensuing months. In adult life, the hemoglobin pattern is normal, and HbH inclusions are not found at any stage. Hemoglobin level and blood film are normal, although the MCV and MCH may be mildly reduced.

Alpha-thalassemia 1 (α-thalassemia): This represents the heterozygous state for a° (– –/αα) or the homozygous state for α⁺-thalassemia (–α/–α). In the neonatal period, 5–6% Hb-Barts is found, but the hemoglobin pattern is normal in later life. HbH inclusions are usually present in very small numbers, and a prolonged search may be necessary for their detection. The hemoglobin level is normal or only mildly reduced, but the red cells are usually mildly hypochromic and microcytic, and the MCV and MCH are reduced. HbA_2 is reduced in some patients.

HEMOGLOBIN H DISEASE

Interaction of the α⁺ (–α) and the α°(– –/1) determinants gives rise to this form of α-thalassemia (–α/––) which is common in South-East Asia and is also seen in the Middle-East and some Mediterranean countries. It is rare in Blacks.

Clinically, HbH disease is characterized by a moderate anemia with α-hemoglobin level of 8–9 g/dL, mild jaundice, and physical findings similar to, but generally less severe than, those of classical β-thalassemia major. The severity of the anemia fluctuates, and it may fall to very low levels during pregnancy, intercurrent infection, or ingestion of oxidant drugs.

Occasionally, the hemolysis is well compensated and the hemoglobin level is normal. Splenomegaly is present in 85% of patients, and cholelithiasis is common.

The *peripheral smear* shows profuse red cell morphological changes including severe hypochromia and microcytosis, target cell formation, and basophilic stippling (Fig. 9.6). The MCV and MCH are low. Normoblasts are seen, and there is mild reticulocytosis. Numerous HbH inclusions may be demonstrated with brilliant cresyl blue stain, and large Heinz body-like inclusions are also present if splenectomy has been performed.

The *hemoglobin pattern* consists of 2–40% HbH, the remainder being HbA, HbA_2 (which is reduced) and HbF. A small amount of Hb-Barts is present in some cases. In South-East Asia, 40% of patients with HbH disease also have a small amount of an α-chain variant, Hb constant spring. Neonates with HbH disease have about 25% Hb-Barts, and only very small amounts of HbH, but in most cases HbH gradually replaces Hb-Barts over the first year of life.

Therapy is not required in most patients with HbH disease. Avoidance of oxidant drugs and prompt treatment of intercurrent infection is advisable. Blood transfusion may occasionally be necessary, and splenectomy has been successful in significantly elevating the hemoglobin in some carefully selected patients with consistently low levels. Folic acid

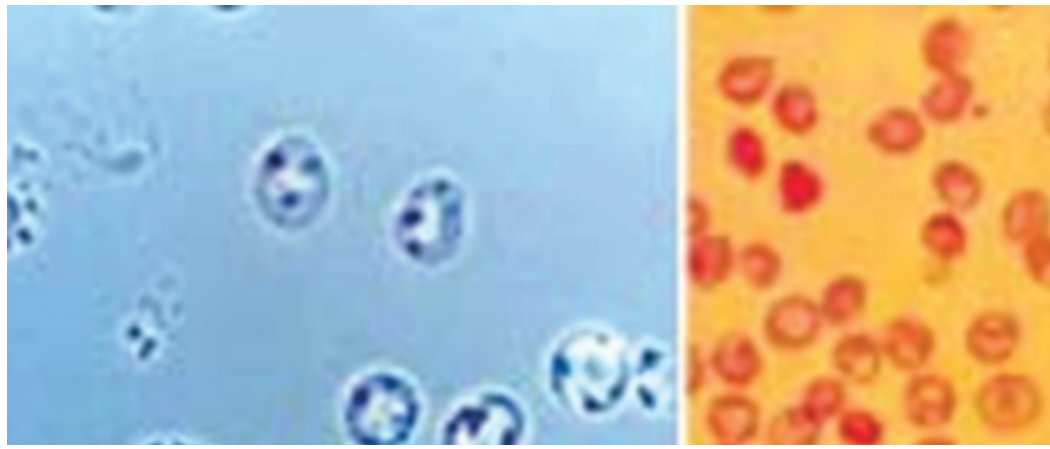


Fig. 9.6: HbH disease, peripheral smear

administration is advisable, especially in pregnancy. Secondary hemochromatosis is rare, and administration of iron-chelating agents is not indicated.

HEMOGLOBIN-BARTS HYDROPS FETALIS

The worst manifestation of the α-thalassemia gene is hemoglobin Barts hydrops fetalis which is common in South-East Asia, but rare in other parts of the world where the α-thalassemia gene is found. Affected infants are homozygous for the α°-determinant (– –/– –), both parents having heterozygous α°-thalassemia. There is almost total suppression of α-chain synthesis with a gross excess of α chains. The γ-chain tetramer, Hb-Barts, has a high oxygen affinity, and severe tissue hypoxia results.

The *clinical picture* is similar to that of severe Rh hemolytic disease. Affected infants are either born dead or die within few hours of birth. They are underweight, pale, mildly jaundiced, grossly edematous, and have hapatosplenomegaly and ascites. The hemoglobin level is around 6 g/dL, and the blood film is grossly abnormal with anisopoikilocytosis, hypochromia, target cells, basophilic stippling, polychromasia, and large number of normoblasts. The reticulocyte count is high, and serum bilirubin elevated. The mother often has an associated toxemia of pregnancy. Antenatal diagnosis of pregnancies at risk for homozygous α-thalassemia is now possible.

The *hemoglobin pattern* consists of 80–90% Hb-Barts, with a small amount of HbH and a third 'fast' component, Hb-Portland. There is usually no HbA, HbA_2 or HbF.

ALPHA-THALASSEMIA AND OTHER HEMOGLOBINOPATHIES

Alpha-thalassemia is found in association with α-chain hemoglobin variants, e.g., HbQ and HbI; β-chain variants, e.g. HbE, HbS, and HbC; and with β-thalassemia.

METHEMOGLOBINEMIA

Methemoglobin (ferrihemoglobin) is a derivative of normal hemoglobin (ferrohemoglobin) in which the iron of the heme complex has been oxidized from the ferrous to the ferric form. It does not combine with oxygen and thus does not take part in oxygen transport. In normal red cells, methemoglobin is continually being formed by the auto-oxidation of hemoglobin, but it is reduced as soon as it is formed; thus the concentration of methemoglobin in the red cell under normal conditions is less than 1% of the total hemoglobin. The reduction of methemoglobin is accomplished by the enzyme NADH–methemoglobin reductase (NADH-cytochrome β_5 reductase) in the presence of NADH. The NADH is generated by the Embden–Meyerhof pathway of glycolysis.

The term methemoglobinemia is used to describe the excess accumulation of methemoglobin in the red cells. Methemoglobin lacks the capacity to carry oxygen, and methemoglobinemia causes signs and symptoms of hypoxia. The great majority of cases of methemoglobinemia are due to the action of chemical agents that increase the rate of auto-oxidation of hemoglobin in the red cells. Rare cases are due to congenital metabolic defects of the red cell.

TOXIC METHEMOGLOBINEMIA

The causes of toxic methemoglobinemia may be classified as given below.

Drug Causes

Drugs which may cause methemoglobinemia include phenacetin, acetanilide, sulphonamides, sulphones, prilocaine, primaquine and pamaquine, nitrates, nitrites and potassium chlorate.

Occupational Causes

Methemoglobinemia in industry is most commonly due to absorption of nitro and amino aromatic derivatives, e.g. nitrobenzene, trinitrobenzene, and aniline. The substances are usually absorbed through the respiratory tract or skin, and the disorder is most often seen in workers in chemical factories and explosive plants.

Household Causes

Chemicals capable of producing methemoglobinemia are present in a number of household substances; these include furniture and shoe polish (containing nitrobenzene), marking ink, shoe dyes and colored crayons (containing aniline), perfumes, and flavoring essences. Acute methemoglobinemia has also been reported as a result of the use of aniline containing inks to mark infants' napkins. Well water sometimes contains high concentrations of nitrates, and in country areas the use of this water to prepare milk mixtures for infants has resulted in methemoglobinemia.

Clinical Features

Cyanosis occurs when methemoglobin constitutes about 15% of the total pigment. In many cases, there are no clinical features other than cyanosis, but when the concentration of methemoglobin reaches 30–45% anoxic symptoms commonly develop. These include headache, dizziness, tachycardia, dyspnea on exertion, muscular cramps, and weakness. In cases of acute poisoning, the concentration may exceed 60–70%, and vomiting, lethargy, loss of consciousness, circulatory failure, and death may occur. In acute cases, the cyanosis develops within 1–2 hours of the ingestion of the toxic agent.

In chronic methemoglobinemia, a mild compensatory polycythemia occasionally develops. Many of the substances that cause methemoglobinemia may also cause a hemolytic anemia with Heinz body formation.

Diagnosis

Clinically, the diagnosis of methemoglobinemia is suggested by the presence of definite cyanosis with a little or no dyspnea. Methemoglobin can be identified spectroscopically by its absorption band in the red part of the spectrum at 630 nm; this band disappears on the addition of yellow ammonium sulfide. When it is present in large amounts the blood has a chocolate-brown color which does not disappear on oxygenation. Methemoglobin does not appear in the plasma or urine except in the occasional case with associated hemolytic anemia.

Therapeutics

Following removal of the causative agent, methemoglobin is converted back to hemoglobin in a few days. Thus, in chronic cases elimination of the causative drug or chemical results in disappearance of the cyanosis within several days.

In cases of acute methemoglobinemia due to poisoning, especially when symptoms are severe, more active treatment is necessary. Treatment consists of slow intravenous injection of methylene blue; the recommended dose is 2 mg/kg body weight for infants, 1.5 mg/kg body weight for older children, and 1 mg/kg body weight for adults, in a 1% sterile aqueous solution.

HEREDITARY METHEMOGLOBINEMIA

Hereditary methemoglobinemia is rare. Two main types are recognized which differ in their fundamental effect and mode of inheritance.

Hereditary methemoglobinemia associated with NADH-methemoglobin reductase deficiency: This disorder is transmitted as an autosomal recessive trait. Affected individuals are persistently cyanotic and usually have mild polycythemia. Some have symptoms of anoxia. Mental retardation is an occasional association. Regular oral ascorbic acid therapy relieves the cyanosis and other symptoms when present.

Hereditary methemoglobinemia associated with hemoglobin-M. Several abnormal hemoglobins, referred to as hemoglobin-M, are associated with cyanosis. The disorder is transmitted as an autosomal dominant trait, and anoxic symptoms are usually absent. The cyanosis is not improved by the administration of methylene blue or ascorbic acid.

SULFHEMOGLOBINEMIA

Sulfhemoglobin is an abnormal sulfur-containing hemoglobin derivative allied to methemoglobin. It does not act as an oxygen carrier and is not present in normal red cells. It is formed by the toxic action of the drugs and chemical agents that cause methemoglobinemia in persons who are either constipated or who are taking sulfur-containing medicines. A history of ingestion of sulfur-containing purgatives such as magnesium sulfate is not uncommon, and in occasional cases there is an associated anatomical abnormality of the bowel, e.g. stricture or diverticulosis. Methemoglobinemia and sulfhemoglobinemia are often present together.

Sulfhemoglobin represents an irreversible change in the hemoglobin pigment; thus it does not disappear from the red cells once the causative agent is removed, but persists until the cells are destroyed at the end of their life span, progressively disappearing from the blood over a period of about three months. Furthermore, it is not converted to hemoglobin by either ascorbic acid or methylene blue.

Sulfhemoglobinemia results in cyanosis, similar to that of methemoglobinemia. Diagnosis is established by spectroscopic examination and isoelectric focusing. Sulfhemoglobin occurs only in the red cells; it is not present in the plasma unless hemolysis occurs.

Treatment consists of removal of the causative agent and correction of constipation when present.

UPTODATE ADVANCES IN HEMOGLOBINOPATHY SCREENING AND DIAGNOSIS

Various methods for hemoglobinopathy newborn screening and adult testing are internationally. Many programs traditionally used electrophoresis as the method of choice for identification and quantification of aberrant hemoglobins. In more recent years, with the introduction of universal screening of newborns, technology has emerged that is robust with high throughput and greater sensitivity over electrophoresis. Many of the same methods used for screening, such as isoelectric focusing (IEF) and high performance liquid chromatography (HPLC) are also used for diagnosis. Some laboratories employ one or more methods in their protocol for detection of hemoglobinopathies. Choice of method depends on the function of the laboratory and its testing algorithm. Molecular methods are emerging and are being utilized by some screening and diagnostic labs alike to obtain a more comprehensive analysis and determine the nature of the hemoglobinopathy and its clinical implications. Below are descriptions of methods used. Descriptions include traditional methods requiring a hands-on approach, more current procedures requiring automation and lastly, molecular assays.

Isoelectric Focusing (IEF)

IEF separates proteins in a gel medium that has a pH gradient consisting of ampholytes (zwitterions). When a high voltage is applied, narrow buffered zones are created with stable, but slightly different pHs (Fig. 9.7). Slower moving proteins migrate through these zones and stop at their individual isoelectric points (pI). In the case of hemoglobin, these migrate to a zone in the medium where the pH of the gel matches the hemoglobin's pI. At the pI, the charge of the hemoglobin becomes zero and ceases to migrate. The hemoglobin migration order of IEF is similar to alkaline electrophoresis. Resolution is clear with differentiation of HbC from HbE and HbO and HbS from HbD and HbG respectively. HbA and HbF are also clearly differentiated. With neonatal specimens, a distinct band representing acetylated HbF is readily identified and slightly anodic to HbA. Staining may be necessary depending on the manufacturer's recommendations. Gels may be read wet or dry and band identification is accomplished by comparison to migration patterns of known quality controls. This method allows for greater precision and accurate quantification than standard electrophoresis. It gives excellent resolution in addition to high throughput, but is also labor-intensive and time-consuming. An alternative method is needed to confirm or differentiate hemoglobin variants (bands other than HbF or HbA). Fast migrating bands (Hb Barts, HbH) can also be identified by IEF.

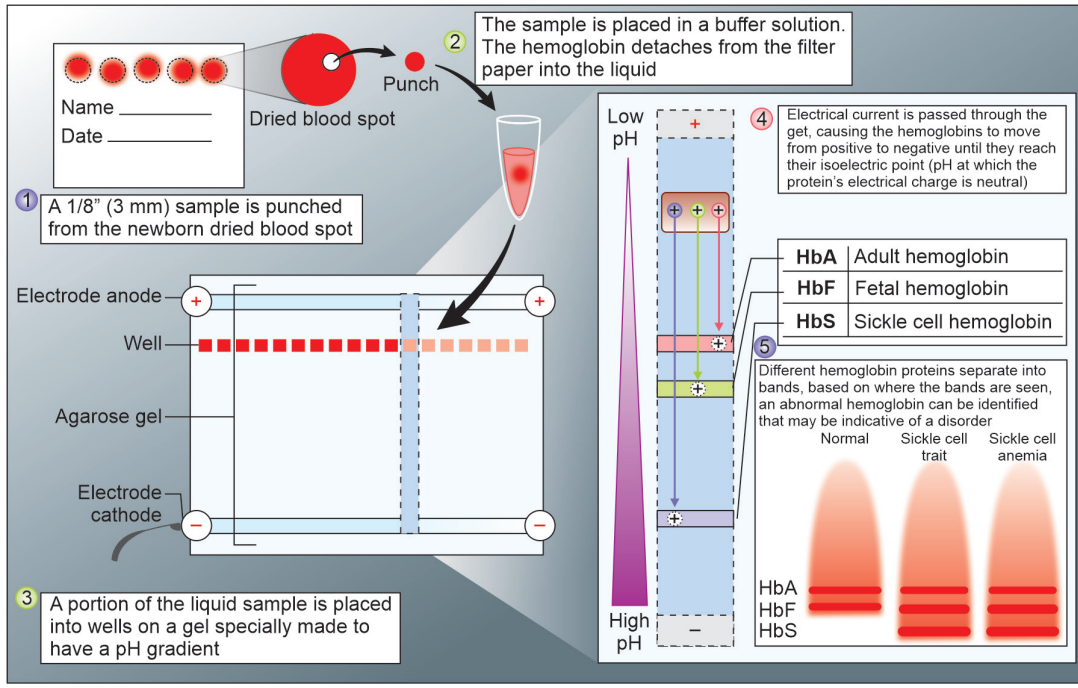

Name
Date
Dried blood spot
Punch
2 The sample is placed in a buffer solution. The hemoglobin detaches from the filter paper into the liquid
1 A 1/8" (3 mm) sample is punched from the newborn dried blood spot
Electrode anode
Well
Agarose gel
Electrode cathode
3 A portion of the liquid sample is placed into wells on a gel specially made to have a pH gradient
Low pH
High pH
4 Electrical current is passed through the get, causing the hemoglobins to move from positive to negative until they reach their isoelectric point (pH at which the protein's electrical charge is neutral)
HbA Adult hemoglobin
HbF Fetal hemoglobin
HbS Sickle cell hemoglobin
5 Different hemoglobin proteins separate into bands, based on where the bands are seen, an abnormal hemoglobin can be identified that may be indicative of a disorder
Normal
Sickle cell trait
Sickle cell anemia
HbA
HbF
HbS

Fig. 9.7: Isoelectric focusing

High Performance Liquid Chromatography (HPLC)

Hemoglobins are separated by an analytical cartridge in cation exchange HPLC using a preprogrammed buffer gradient with increasing ionic strength to the cartridge (Fig. 9.8). The hemoglobin fractions separate based on their ionic interaction with the cartridge. The separated fractions pass through a flow cell, where absorbance is measured

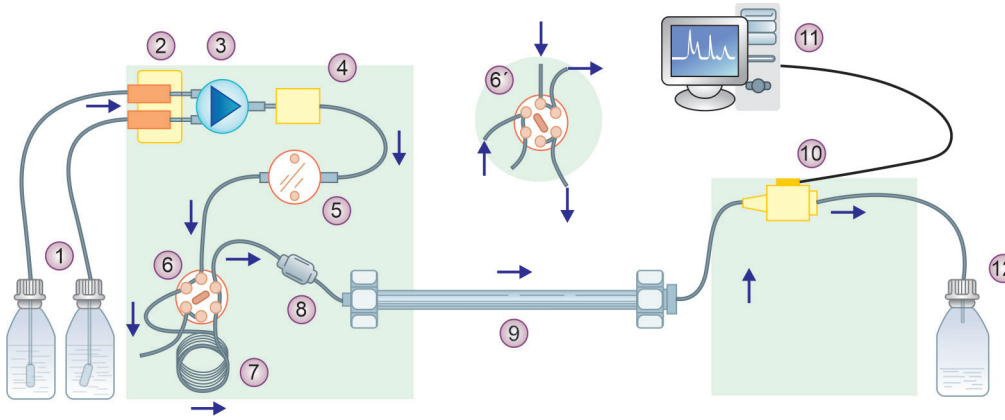


Fig. 9.8: High performance liquid chromatography

Schematic representation of an HPLC unit. (1) Solvent reservoirs, (2) solvent degasser, (3) gradient valve, (4) mixing vessel for delivery of the mobile phase, (5) high-pressure pump, (6) switching valve in "inject position", (6') switching valve in "load position", (7) sample injection loop, (8) pre-column (guard column), (9) analytical column, (10) detector (i.e. IR, UV), (11) data acquisition, (12) waste or fraction collector.

at 415 nm and again at 690 nm to reduce background noise. Changes in absorbance are monitored over time producing a chromatogram (absorbance vs. time). Each hemoglobin has its own characteristic retention time and is measured from the time of sample injection into the HPLC to the maximum point of each peak. Identification of unknown hemoglobin is achieved through comparison with known hemoglobin retention times. If a peak elutes at a retention time not predetermined, it is labeled as an unknown. HPLC achieves good separation and quantitation of HbF and HbA in addition to screening for variant hemoglobins along with thalassemia. HPLC is highly reproducible, offers simplicity with automation, superior resolution and rapid results. Some HPLC instrument programs can identify hemoglobinopathies from both newborns and adult specimens while others identify only one or the other. Identification between adult and newborn specimens depends on the algorithm/software/instrument specifications.

The following methods are used less frequently than those described above. Some methods have a long history within laboratories and continue to be used to clarify and confirm abnormal hemoglobins. Newer technology, like molecular methods, although utilized by a few programs, is gaining momentum with some programs.

Cellulose Acetate Electrophoresis (Alkaline)

Cellulose acetate electrophoresis or alkaline electrophoresis makes use of the negative charge which hemoglobin will adopt under alkaline conditions. Samples are applied to cellulose acetate agar gel and hemoglobins are separated by electrophoresis using an alkaline buffer (tris-EDTA with boric acid) at pH 8.4. Each hemoglobin variant carries a different net charge so it will migrate at varying speeds. Following electrophoretic migration, visualization of hemoglobin bands are accomplished through staining with Ponceau S, Amino Black and acid violet or other similar stains and compared with known standards. Hemoglobins are quantified using densitometric scanning and the relative percentage of each band is determined. This method yields rapid and reproducible separation of HbA, HbF, HbS and HbC as well as other variant hemoglobins with minimal preparation time. However, due to limited sensitivity and because some hemoglobins are

electrophoretically similar though structurally different, an alternative procedure must be incorporated into the screening algorithm for differentiation of these hemoglobins. For example, HbS, HbD, HbG and Hb-Lepore co-migrate, so they are indistinguishable on alkaline electrophoresis. The same is true for HbC, HbA_2, HbO-Arab and HbE.

Citrate Agar Electrophoresis (Acid)

Electrophoresis occurs in an acidic environment at pH 6.2. This method is based on the complex interactions of the hemoglobin with the electrophoretic buffer and the agar support. Staining allows visualization of hemoglobin bands. Acid electrophoresis allows confirmation of variant hemoglobins observed in the cellulose acetate electrophoresis procedure and allows good separation of HbC from HbE and HbO-Arab. It permits additional separation of HbS from HbD and HbG. Citrate agar electrophoresis appears to be more sensitive than cellulose acetate for detecting HbF.

Alkaline Globin Chain Electrophoresis

The hemoglobin molecule can be separated into its globin chain components and heme groups by the addition of 2-mercaptoethanol and urea. At alkali pH, these globin chains will migrate to their characteristic position on a cellulose acetate strip, under an electrical field. The characteristic migration pattern aids in the identification of each globin chain type.

Capillary Zone Electrophoresis

Charged molecules are separated by their electrophoretic mobility in an alkaline buffer (pH 9.4) with separation occurring according to the electrolyte pH and endosmosis or electro-osmotic flow. Capillaries function in parallel allowing eight simultaneous analyses. Samples are hemolyzed and injected into the anodic end of the capillary. High voltage protein separation is performed and hemoglobins migrate from the anodic end of the capillary appearing in specific zones to the cathodic end where detection occurs at 415 nm. Results are assessed visually for abnormalities with identification of normal and disease patterns. There is improved detection of sickle cell disease due to separation of hemoglobin fractions which enables differentiation of HbS from other variants. Capillary zone electrophoresis allows clean separation of HbE from HbA_2 and facilitates easier detection of Hb-Barts and HbH (Figs 9.9 to 9.11)

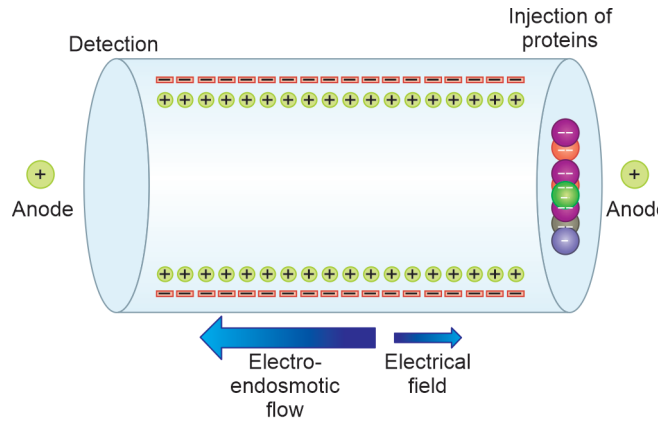


Fig. 9.9: Principle of hemoglobin electrophoresis using capillary electrophoresis technologies

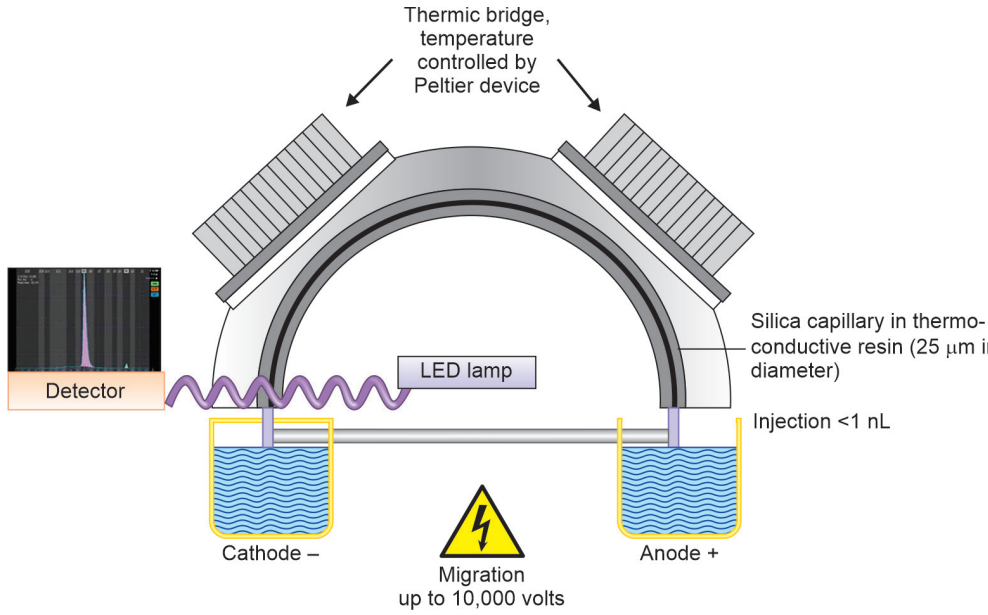


Fig. 9.10: Capillary diagram

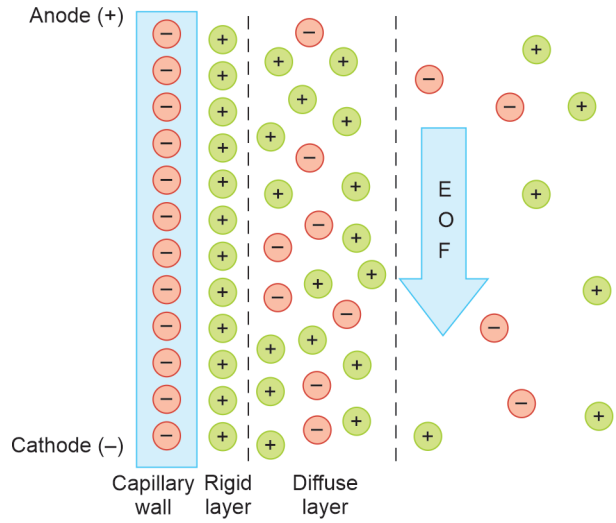


Fig. 9.11: Net flow of buffer toward the negative electrode (cathode)

Molecular Methods

Molecular testing for hemoglobinopathies generally uses three techniques. These are restriction fragment length polymorphism (RFLP), allelic discrimination using real-time PCR end point data and DNA sequencing. DNA extraction of whole blood in the DBS filter paper matrix is needed for all PCR-based assays. Methods for DNA extraction include crude boiling preparations, alkaline lysis preparations and commercial methods. Each one has its own benefits and limitations, and each laboratory needs to determine the best method for their applications.

Considerations for all Polymerase Chain Reaction (PCR)-based Assays

All molecular assays using PCR are susceptible to contamination by aerosolized amplicons. To minimize the risk of contamination, at a minimum, assay set-up and amplification/analysis must be separated and one-way directional work flow is required. Depending upon the volume of specimens tested and nature of the work, laboratories may require shoe covers and hair caps in addition to a laboratory coat and gloves.

Each PCR run must include a positive control (a genotype positive DBS or genomic DNA, i.e. SS, SC, AS, AC, etc.) and a negative control, also referred to as a no template control (no DNA control, i.e. water or reagent only). The positive control verifies that the amplification occurred and the negative control will detect contamination of reagents.

Restriction Fragment Length Polymorphism (RFLP)

RFLP takes advantage of recognition sequences of restriction enzymes that correspond to the normal allele or the mutated allele of a gene. If the recognition sequence is present, the restriction enzyme will restrict (cut) the DNA and the size difference can be visualized by gel electrophoresis (Fig. 9.12).

To use RFLP, DNA is first extracted from DBS and amplified using polymerase chain reaction (PCR). The amplified DNA is added to the restriction enzyme reaction and incubated for a prescribed amount of time. An aliquot of the mixture is electrophoresed and the size(s) of the fragment(s) is visualized after staining, photographed and analyzed. For example, the S mutation in the beta-globin B (HBB) gene replaces an adenine with a thymine resulting in an amino acid change from a glutamic acid to valine. This change destroys the DdeI restriction enzyme recognition site. After amplifying a 125 bp fragment of the HBB gene, an aliquot of the PCR reaction is added to a mixture containing the enzyme DdeI. After a prescribed amount of time, an aliquot of this reaction and a DNA molecular weight marker or DNA ladder is electrophoresed on an agarose gel. If the specimen has normal hemoglobin (HbAA), the enzyme cuts the DNA into 106 bp and 19 bp fragments. The 106 bp band is visible, but the 19 bp band is too small to see on the gel. If the only product seen is 125 bp, both alleles have the S mutation (SS). The specimen would be considered to have both an A and S allele (AS) and is classified as a carrier if both the 125 and 106 bp bands are seen (Fig. 9.12).

The benefits of this method are that it is very easy to perform and analyze and is inexpensive compared to the other molecular methods described below. The only instrumentation required is a PCR machine, a water bath, an electrophoresis unit and a camera. It is a low throughput method because there are a number of manual steps and the amount of time it takes to complete the assay; however, it is a good choice for laboratories that have a small number of samples to test. A limitation of RFLP is that partial restriction (incomplete cutting) of the DNA could result in a homozygous specimen being interpreted incorrectly. A positive control of a known genotype is crucial to identify this problem. In cases of a double mutation like SC, if the restriction enzymes do not have similar activity conditions, two separate reactions are required.

Allelic Discrimination using Real-time PCR

Allelic discrimination (AD) measures fluorescence at the end of PCR to determine if a mutation is present. For this technique, a forward and reverse primer spanning the area of interest are used as well as hydrolysis probes. Hydrolysis probes are oligonucleotides that have a fluorophor bound to the five prime (5′) end and a quencher molecule bound to the three prime (3′) end. When the quencher is in close proximity to the fluorophor, fluorescence cannot be detected. After DNA is extracted from the specimen, a PCR reaction

with the forward and reverse PCR primers, DNA polymerase, nucleotide triphosphates, (dNTPs) and two probes is prepared. One probe corresponds to the normal sequence and the other corresponds to the mutant sequence. The fluorophor bound to each primer emits fluorescence at different wavelengths and can be distinguished. During PCR, the primers and probes bind to their complementary sequences. When DNA polymerase encounters the bound probe, the polymerase's 5′–3′ exonuclease activity degrades it. The fluorescent molecule is released, separating it from the quencher and the fluorescence can be measured (Fig. 9.7). Because amplification occurs exponentially, there is a large increase of fluorophors that are no longer in proximity to the quenchers. At the end of the amplification, the fluorescence is measured in a real-time PCR machine and the results are analyzed by the software. If the signal from only one probe is detected, the sample is homozygous for the sequence complementary to that specific probe. If the signals from both probes are detected, then the sample has both a mutant allele and a normal allele and is considered a carrier (Fig. 9.13).

This method has a higher throughput than RFLP because the results are generated right after the amplification ends, with no further processing. All reactions take place in the PCR tube which never has to be opened, thus reducing the risk of contamination. The throughput can be increased by using 96- or 364-well PCR plates and automation. Limitations include an increased cost for the probes and the need for a real-time PCR machine. Although AD is considered a multiplexed assay, the limited number of filters in the PCR machines does not allow for a higher order of multiplexing.

DNA Sequencing

Sanger sequencing is the most comprehensive method of mutation detection and determines the exact sequence spanning the area of the primers used. Once DNA is extracted from the sample, it is amplified by PCR but in addition to the primers, the DNA polymerase and dNTPs, dideoxynucleotides (ddNTPs) are added. Each of the four ddNTPs has a specific fluorophor bound to it, which emits a signal at a different wavelength. During amplification, when one of the ddNTPs is incorporated, extension stops because the lack of a hydroxyl group does not allow for the addition of the next base. The ratio of dNTPs to ddNTPs is optimized to generate fragments that are one base different than the next. After amplification, the products are purified, typically by DNA precipitation or by spin column. They are loaded onto a capillary electrophoresis instrument that separates the products by size based on their charge. When the products move across the path of a laser in the instrument, each fluorophor emits a signal at a different wavelength and this information is captured, analyzed by the instrument software and the sequence is displayed (Fig. 9.14).

The greatest benefit of this method is that every base change in the area of interest is determined including single nucleotide polymorphisms, small insertions and small deletions. Sequencing is a high-throughput method but all the sequence data must be reviewed by a trained technician, which can take a large amount of time depending on the length of sequence and the number of samples. For newborn screening laboratories, if sequencing is used for hemoglobinopathy screening, only the betaglobin gene, HBB, is usually sequenced because it is relatively small and the most common mutations tested are point mutations in HBB. The α-globin genes, HBA_1 and HBA_2, have >96% sequence homology making sequencing of these genes more complicated. Many of the common α-thalassemia mutations are large deletions which cannot be found by sequencing but can be found using gap PCR or multiplex ligation-dependent probe amplification. Sequencing is the most expensive method compared to RFLP and AD because of the reagents,

instrumentation, personnel, and review time required for analysis, but it provides the most comprehensive data for the β-globin gene.

Programs use different approaches for hemoglobinopathy screening, confirmation and diagnosis.

Newborn screening programs however do not routinely screen for the purpose of detecting α or β-thalassemias although screening methods may detect some forms of these hemoglobinopathies. Clinical presentation in conjunction with hematologic features exhibited in the complete blood count (CBC) and peripheral blood smear as well as iron studies aid in confirmation and diagnosis of thalassemias. Depending on the type of thalassemia diagnosed, family studies may be warranted along with genetic counseling.

To further improve screening and diagnosis of hemoglobinopathies including thalassemias, some laboratories are exploring the use of tandem mass spectrometry and whole genome sequencing. These methods are promising improved sensitivity and specificity for hemoglobinopathies. However as procedures advance, others become less ideal for screening. For example, HbS solubility test, although simple and easy to use, is characterized by false-positives from other hemoglobin types and interfering substances that may be present in the sample. False-negatives are commonly seen in patients with low hemoglobin or hematocrit levels. Other abnormal hemoglobins are not readily detected and result interpretation can be difficult due to the subjectivity of the test. Lastly, the HbS solubility test cannot differentiate between carrier and disease state for sickle cell disease. Based on these limitations, solubility testing is not recommended.

Additionally, when deciding on which method is most appropriate for hemoglobinopathy detection, it is necessary to consider the benefits the method will bring to the individual program. Likewise, it is important to consider expense, throughput, ease of use and skills required for implementation and routine analysis. Methods also have different limitations that must be evaluated. For example, peak or band resolution, differentiation of hemoglobins and result interpretation are important limitations to evaluate. The next section explores some of the limitations of the more commonly used applications.

Examples of Molecular Methods for Hb Mutation Detection

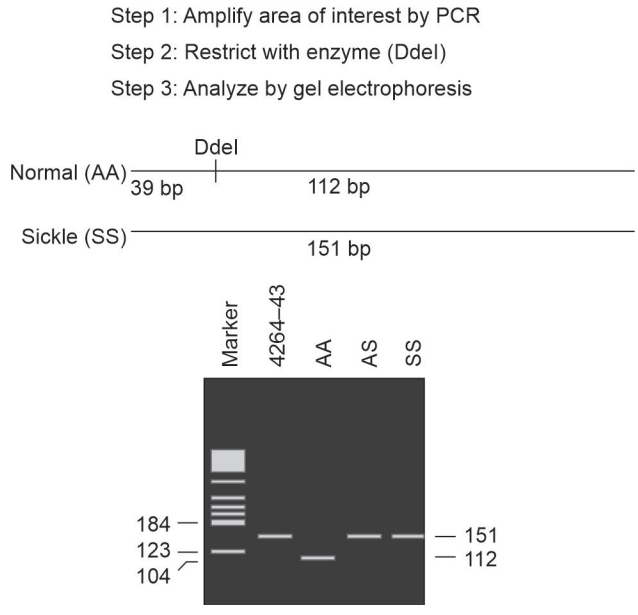


Fig. 9.12: PCR-RFLP

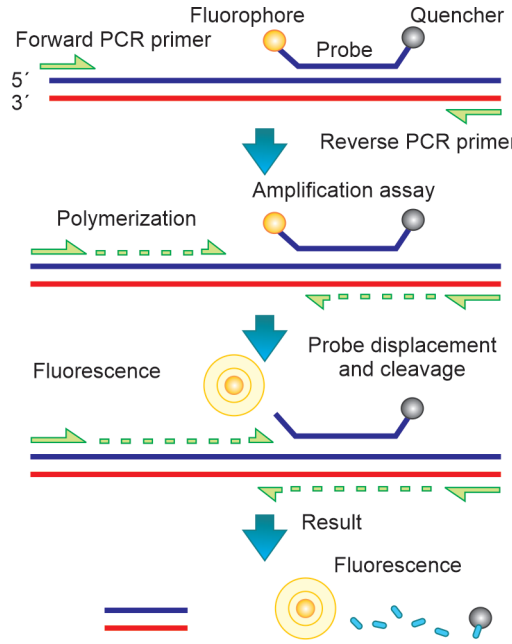


Fig. 9.13: Allelic discrimination

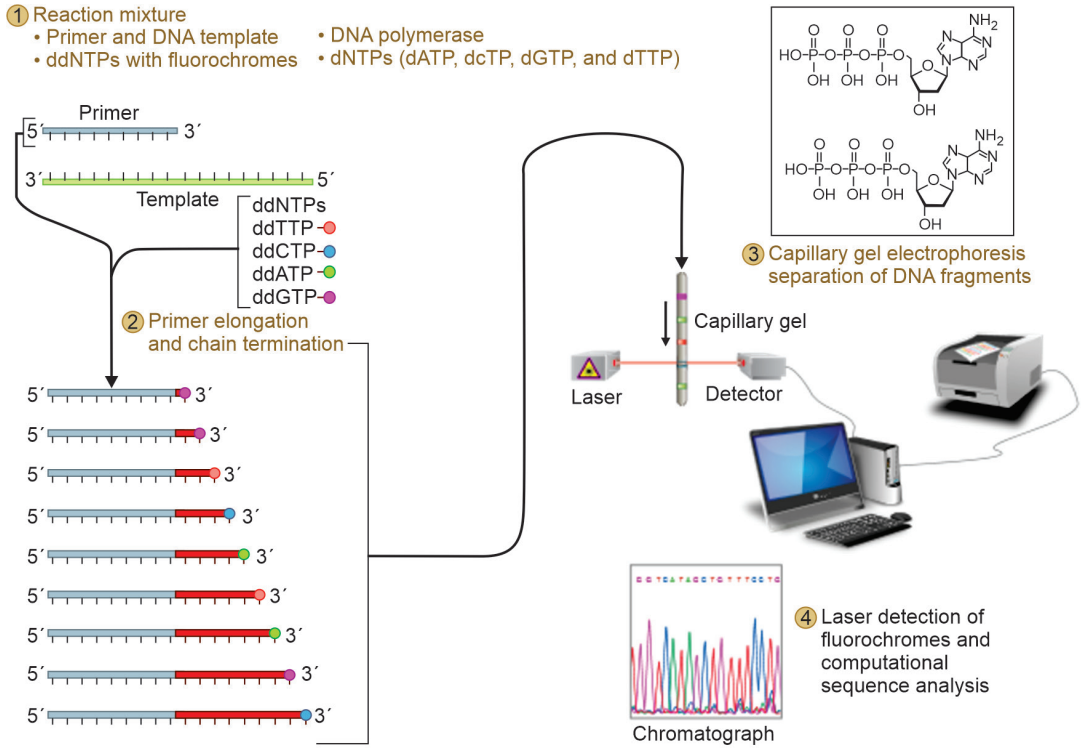


Fig. 9.14: Sanger sequencing

CHAPTER

10

Aplastic Anemia, Pancytopenia

PANCYTOPENIA

Concurrent presence of anemia, leukopenia, and thrombocytopenia. Pancytopenia, leukopenia, and thrombocytopenia is referred to as pancytopenia. Pancytopenia therefore exists in an adult when the hemoglobin level is less than 13.5 g/dL in males, or 11.5 g/dL in females; the leukocyte count is less than 4×10^9/L; and the platelet count is less than 150×10^9/L.

Patients present with symptoms are usually attributable to the anemia or the thrombocytopenia. Leukopenia is an uncommon cause of the initial presentation of the patient, but can become the most serious threat of life during the subsequent course of the disorder. Sometimes, pancytopenia is detected as an incidental feature in a patient who has presented with symptoms of a disorder that is capable of depressing the levels of all cellular elements in the blood. A wide variety of disorders can cause pancytopenia, as indicated in Table 10.1, although the frequency with which each condition is associated with pancytopenia differs considerably. However, pancytopenia is essentially always present at some stage in the course of aplastic anemia, very common in subleukemic

Table 10.1: Causes of pancytopenia

- Aplastic anemia
- Subleukemic acute leukemia
- Administration of cytotoxic agents and antimetabolites
- Radiotherapy
- Myelodysplastic disorders
- Bone marrow infiltration or replacement:
  - Hodgkin's and non-Hodgkin's lymphoma, macroglobulinemia
  - Multiple myeloma
  - Metastatic carcinoma in bone marrow
  - Myelofibrosis
- Hypersplenism
- Megaloblastosis—vitamin B_{12} and folate deficiency
- Systemic lupus erythematosus
- Paroxysmal nocturnal hemoglobinuria
- Overwhelming infection
- Miscellaneous

leukemia, relatively uncommon in lymphoma, and rare in metastatic carcinoma involving the bone marrow. The prognosis depends both on the severity of the pancytopenia and on the nature of the underlying condition.

ETIOLOGY

1. Disorders infiltrating the bone marrow
   a. Aleukemic/subleukemic leukemia
   b. Multiple myeloma
   c. Metastatic carcinoma, lymphoma
   d. Myelofibrosis, myelosclerosis, agnogenic myeloid metaplasia
   e. Marble bone disease, osteopetrosis
2. Disorders involving the spleen
   a. Congestive splenomegaly, hypersplenism
   b. Lymphomas: Hodgkin's disease, non-Hodgkin's lymphoma.
   c. Infiltrative disorders: Gaucher's disease, Niemann–Pick disease, Letterer–Siwe disease
   d. Infectious diseases: Kala-azar, miliary tuberculosis, syphilis
   e. Primary splenic panhematopenia
3. Vitamin B_{12} or folate deficiency, in pernicious anemia. sprue, etc.
4. Disseminated lupus erythematosus
5. Paroxysmal nocturnal hemoglobinuria
6. Miscellaneous disorders (sometimes)
   - Overwhelming infections
   - Mycobacterial infections
     - Brucellosis
     - Sarcoidosis
     - Pregnancy
   - Some refractory anemias
   - Sideroblastic anemia
     - Drug sensitivity
7. Aplastic or hypoplastic anemia

DIAGNOSIS OF PANCYTOPENIA

Clinical presentation is based upon features of pancytopenia *per se*, and those of the causative disorder. Sometimes, the clinical picture and examination of the blood readily indicate the nature of the causative disorder. The major diagnostic problems occur when there are no specific features in the blood to suggest the diagnosis, or when the clinical features are not sufficiently specific to point to the cause of an associated feature such as splenomegaly or lymphadenopathy.

INVESTIGATION OF CASES WITH PANCYTOPENIA

Clinical presentation usually indicates the aetiology. Blood examination, and examination of the bone marrow aspirate and trephine biopsy assist further. When these data do not establish the diagnosis, further investigations are necessary. The nature and order of these investigations vary with the provisional diagnosis, and are appropriate for establishing the presence or absence of the disorders listed in Table 10.2. Certain biochemical investigations can, for instance, be helpful. Detection of an elevated serum lysozyme concentration, for example, in a patient with pancytopenia is indicative of an underlying myeloid neoplastic

Table 10.2: Outline of details required in the investigation of a patient with pancytopenia

History
- Age, sex, occupation, diet
- Exposure to chemicals, drugs, or radiation—bone pain
- Fever, night sweats, malaise, weight loss, pruritus
- Symptoms of disorders causing major splenic enlargement

Physical examination
- Lymph node enlargement—splenomegaly
- Bone tenderness, deformity, or tumor—hepatomegaly
- Gum hypertrophy
- Signs of disorders causing hypersplenism, especially portal hypertension
- Evidence of primary malignancies often associated with metastasis to bone, especially breast, prostate and lung

Laboratory investigations
- Essential investigations in all cases:
- Peripheral blood examination. Especially note:
  - Anisocytosis and poikilocytosis
  - White and red cell precursors
  - Abnormally increased or decreased granulation in neutrophils
  - Hypo- or hypersegmentation in neutrophils
  - Erythrocyte rouleaux formation
  - Erythrocyte sedimentation rate
- Bone marrow aspiration and trephine

Further investigations where indicated*:
- Bone X-ray (multiple myeloma, metastatic carcinoma, lymphomas)
- Chest X-ray (lymphomas, carcinoma of the lung, tuberculosis)
- Serum alkaline and acid phosphatase level (metastatic carcinoma)
- Serum protein electrophoresis (multiple myeloma, macroglobulinemia)
- DNA antibody, lupus erythematosus cell test (systemic lupus erythematosus)
- Urinary Bence–Jones protein (multiple myeloma)
- Needle biospy of liver (hypersplenism, lymphomas, disseminated tuberculosis)

* Diseases in which a particular investigation has considerable diagnostic value are bracketed with that investigation.

infiltrative process rather than aplasia of the bone marrow. Occasionally, extensive investigation and/or prolonged observation are necessary before a definite diagnosis can be established.

The features of particular relevance to the investigation of a patient with pancytopenia are listed below. A careful examination of the blood film is often helpful in giving a lead to the diagnosis and, as marrow examination usually establishes the diagnosis, some of the more important points of these investigations are summarized below.

Blood Examination

Anisocytosis and Poikilocytosis

Anisocytosis and poikilocytosis of moderate degree are common in acute leukemia, but are by no means invariably present in this disorder. They are present, but less marked, in aplastic anemia. Both changes may be quite conspicuous in metastatic bone carcinoma. They are usually less obvious in marrow infiltration by lymphomas or multiple myeloma. Poikilocytosis is often very marked in myelofibrosis, pear and tearshaped poikilocytes being especially significant.

White and Red Cell Precursors

These are almost invariably present in myelofibrosis as a relatively small proportion of the total nucleated cells in the blood. Such a leukoerythroblastic picture is also common in subleukemic leukemia and metastatic carcinoma in bone. It is less characteristic but can occur in multiple myeloma and marrow involvement by lymphoma. It is not typical of aplastic anemia, so that its presence in pancytopenia suggests a diagnosis other than aplastic anemia. In subleukemic leukemia and acute myelofibrosis, occasional typical and atypical 'blast' cells can be present and suggest the diagnosis. Immature lymphoid or plasmacytic cells can likewise occur occasionally in lymphoma and multiple myeloma, respectively.

Abnormal Granulation in Neutrophils

Toxic granulation occasionally occurs in aplastic anemia independently of infection, which is the usual cause of this abnormality.

Hyposegmentation or Hypersegmentation in Neutrophils

Hypogranular neutrophils are found in some myelodysplastic disorders and acute non-lymphoblastic leukemias. Pelger-Huet-like cells are seen in myelodysplastic disorders and some leukemias. An increased number of nuclear lobes is not specific for megaloblastosis, but this feature, coupled with macrocytic poikilocytic erythrocytes, strongly points to such a diagnosis.

Peripheral Smears

These may show rouleaux formation of slight to moderate degree in patients with a high sedimentation rate, but the marked rouleaux formation seen in cases of multiple myeloma and macroglobulinaemia is seldom seen in other causes of pancytopenia. Giant platelet forms may be seen in leukemia and myelodysplastic syndromes, but platelet size is normal in aplastic anemia.

Erythrocyte Sedimentation Rate

The sedimentation rate is commonly raised in many of the disorders causing pancytopenia. In aplastic anemia, it is almost invariably raised. In multiple myeloma and macroglobulinaemia, values are commonly very high and may exceed 150 mm/hour; this is seldom seen in other disorders causing pancytopenia.

Bone Marrow Aspiration and Trephine

A 'dry' or 'blood tap' is not uncommon in disorders causing pancytopenia, and repeated attempts may fail to obtain sufficient marrow particles for adequate examination. A marrow trephine biopsy should be routinely performed, as it not only provides information about the cellularity of hemopoietic elements, but also about the presence of reticulin and certain types of abnormal cells which are difficult or impossible to aspirate from the bony cavity. Sometimes the diagnosis can be established only when the procedure is performed at a site of focal bone involvement indicated by localized tenderness, deformity, or radiological abnormality.

Marrow aspiration is usually diagnostic in subleukemic leukemia, multiple myeloma, and aplastic anemia. It is often diagnostic in marrow involvement by caricinoma and

in myelodysplastic disorders. It can be helpful in non-Hodgkin's lymphoma and in macroglo- bulinemia, but a trephine biopsy is necessary to establish the presence of myelofibrosis or involvement by Hodgkin's disease. The marrow in hypersplenism does not show any specific changes, but is usually hypercellular due to active erythropoiesis and leukopoiesis. When disseminated tuberculosis is suspected, a marrow film should be stained by the Ziehl–Neelsen method, and isolation of typical or atypical mycobacteria by culture should be attempted.

APLASTIC ANEMIA

This is not the most common cause of pancytopenia, but it is a serious and usually chronic disorder and is described here in some length. The other conditions causing pancytopenia, as indicated in Table 10.1, are discussed in detail in the relevant sections which deal with each particular disorder.

Introduced in 1888 by Ehrlich, the term aplastic anemia describes a disorder of unknown etiology characterized by anemia, leukopenia, and thrombocytepenia resulting from a aplasia of the bone marrow. The fundamental pathological feature is reduction in the amount of hemopoietic tissue, causing an inability to produce normal numbers of mature cells for discharge into the peripheral blood. Although there is a marked reduction in the total amount of hemopoietic tissue in the bone marrow, the marrow is not always uniformly hypocellular, and patchy areas of normal cellularity or even hypercellularity are sometimes interspersed between the area of hypocellularity.

Administration of cytotoxic agents and antimetabolites, or exposure to substantial amounts of ionizing radiation, also decrease the amount of hemopoietic tissue in the bone marrow, but the defect differs from that in aplastic anemia in that it most commonly undergoes progressive recovery from the time that exposure to the offending agent is terminated. Ingestion of radioactive materials with a propensity to localize in bone can cause pancytopenia, which represents an industrial hazard formerly prevalent in the mining and handling of radioactive materials.

CLASSIFICATION

Aplastic anemia is generally classified as follows:

1. Idiopathic when no cause, or any association with other conditions, is evident.
2. Secondary when the disorder is the result of exposure to certain drugs or chemicals, a sequel to certain viral infections, or related to certain other specific conditions. The most important relationships are with:
   - Drug idiosyncrasy
   - Chemical exposure
   - Infectious hepatitis
   - Pancreatic insufficiency
   - Paroxysmal nocturnal hemoglobinuria
   - Pure red cell aplasia
3. Constitutional when associated with inherited defects in DNA repair as seen in Fanconi's syndrome.

Selective aplasia of erythroid precursors is referred to as pure red cell aplasia, and the two main categories of this disorder exhibit different chemotherapeutic responses from aplastic anemia. The major caterogies are classified as: congenital or Diamond–Blackfan anemia, and acquired either with or without associated thymoma.

ETIOLOGY OF APLASTIC ANEMIA

Despite the diversity of causative relationships, there are no basic differences in the course of the disorder, or the response to therapy. There is no unanimity of opinion as to the etiology of aplastic anemia, and it may represent a common end-result of different toxic mechanisms, which are abetted in some instances by an, as yet unexplained, increase in susceptibility to toxic processes in certain individuals.

A feature of the disorder, apart from in the relatively uncommon familial form, is that it often develops as a response to extrinsic factors, drugs and certain chemicals, which are normally tolerated by the majority of the population. Such idiosyncratic reactions to commonly employed therapeutic agents represent one of the most important causes of aplastic anemia. It is on the other hand clear that the disorder can develop at least as frequently in absence of evident exposure to drugs or chemicals. Such instances are therefore due either to an idiosyncratic response to hitherto unrecognized extrinsic factors, or to a different type of pathological process. The response in aplastic anemia patients who are recipients of transplanted genetically identical bone marrow from a healthy identical twin, indicates the existence in some cases of an ongoing toxic process which can injure hemopoietic tissue. This toxic process can be suppressed by measures that involve potent cytotoxic and immunosuppressive capability, although the precise nature of the mechanism responsible for the injury to hemopoietic tissue remains unexplained.

DRUG IDIOSYNCRASY

Idiosyncratic reactions are qualitatively abnormal reactions to a drug which cause hypoplasia of the bone marrow, and may occur when a drug is first given. The relationship to the daily or total ingested dose is very variable. Toxic effects sometimes appear only after large doses or prolonged courses, but have been reported to occur after small doses or short courses. The risk to any one individual in the population is small, but if the drug is widely used, the number of persons affected can become significant.

Drugs that cause aplastic anemia due to idiosyncrasy or hypersensitivity may be broadly subdivided into 'higher-risk' and 'lower-risk' drugs. With 'higher-risk' drugs, the per capita incidence of individual developing aplastic anemia is relatively small, but nevertheless constitutes a fairly constant proportion of patients receiving the drug. With 'lower-risk' drugs, aplasia occurs only rarely, considerably less than in 1 in 10000 patients treated. Oxyphenbutazone, chlorpromazine, phenylbutazone, gold salts, and chloramphenicol have, overall, been the drugs most commonly linked with aplastic anemia (Table 10.3).

Chloramphenicol produces two distinct patterns of hematological toxicity: dose dependent reversible hemopoietic depression in all individuals, and idiosyncratic aplastic anemia in a small proportion of individuals.

The reversible toxic effect takes the form of an arrest of erythropoiesis and consequent depression of the reticulocyte count when serum levels of chloramphenicol are sustained at or above 15–20 μg/mL for at least several days. Production of platelets can also be depressed under these circumstances, leading to the development of thrombocytopenia. Examination of the bone marrow reveals decreased proportions of erythroid precursors which may also have vacuolated cytoplasm. These changes are normal pharmacological responses to relatively high blood levels of chloramphenicol, and occur in all individuals. Recovery of erythropoiesis and thrombopoiesis normally takes place in a matter of days when the administration of chloramphenicol is stopped.

Table 10.3: Drugs associated with idiosyncratic aplastic anemia	
Anticonvulsants	***Antirheumatic drugs***
Methylhydanton* (H)	Oxyphenbutazone (H)
Trimethadione* (H)	Phenylbutazone (H)
Paramethadione* (H)	Indomethacin
Aloxidone	Gold salts* (H)
Phenacemide*	Diclofenac
Methylphenylhydantion	
Methsuximide	
Antibacterial drugs	***Antidiabetic drugs***
Chloramphenicol (H)	Tolbutamide
Sulfonamides*	Chlorpropamide
Isoniazid	
Arsenicals*	
Tranquillizers	***Miscellaneous***
Meprobamate*	Chlorothiazide*
Pecazine	Mepacrine
Chlordiazepoxide	Hydralazine
Chlorpromazine* (H)	Acetazolamide
Promazine*	Potassium perchlorate
Thioridazine*	Carbamazepine*
	Tripelennamine

*Selective neutropenia or thrombocytopenia more common toxic effects; (H) relatively high-risk drug.

This kind of an effect is not usually observed with conventional therapeutic doses, because the relatively high blood levels required to produce a significant degree of reversible toxicity are not achieved unless detoxification of the drug is impaired, either by hepatic disease or immaturity. This type of reaction is thus normally restricted to individuals receiving relatively high doses such as 50 mg/kg/day. As the response is readily reversible, it is not of serious clinical importance, unless it is not recognized and depression of all hemopoietic elements develops during protracted treatment.

Chloramphenicol causes this effect by depressing cellular proliferation as a result of its ability selectively to inhibit the synthesis of certain mitochondrial proteins normally produced by the protein synthesizing system in mitochondria. The affected proteins include cytochrome oxidase, an essential component of the major energy generating system in the cell. The ensuing lack of energy availability leads to arrest of proliferation, which is evident first in rapidly dividing tissues such as erythroid precursors.

Idiosyncratic aplastic anemia, on the other hand, develops without a specific relationship to the daily or total ingested dosage of chloramphenicol. There may be a history of previous exposure to chloramphenicol, but this is far from inevitable. The majority of instances follow administration of chloramphenicol for several days or more at conventional dosages. Symptoms may not become evident for weeks or months, but the epidemiological evidence is overwhelming that this drug is a cause of aplastic anemia.

Attempts to define the degree of risk in any individual taking a course of chloramphenicol suggest the incidence is approximately ten times greater than the background level of spontaneously occuring idiopathic aplastic anemia. It appears that somewhat fewer than 1 in 10000 individuals develops aplastic anemia when administered chloramphenicol, but such a degree of risk is unacceptable when alternative antibiotics with less serious side-effects are equally satisfactory in eradicating infection. Such widespread administration of chloramphenicol occurred at one point in time that it appeared responsible for an overall increase in the incidence of aplastic anemia, and as a result the drug was withdrawn from general use in some countries. There is currently insufficient epidemiological data to establish whether the few reports of aplasia associated with the use of chloramphenicol responses, or chance associations with a condition that can occur spontaneously.

Consumption of a number of other therapeutic agents such as gold salts and phenylbutazone is associated with an increased incidence of aplastic anemia. A large number of other drugs have been associated in a small number of instances with aplastic anemia, but it is difficult to ascertain whether these represent chance relationships or otherwise. There is currently no evidence of a mechanism to account for the idiosyncratic response to any drug. Efforts to identify increased drug sensitivity in proliferating cultured myeloid progenitors from subjects who have had aplastic anemia have in fact demonstrated the opposite finding of reduced susceptibility to the toxic action of the incriminated drug in the case of chloramphenicol and phenylbutazone.

CHEMICAL EXPOSURE

The most significant chemical associated with aplastic anemia is benzene. This is a cyclic hydrocarbon and should not be confused with petroleum. Three factors influence the development of aplastic anemia: susceptibility of the individual, duration of exposure, and concentration of vapor.

Aplastic anemia is usually produced by inhalation of benzene vapour for considerable periods, as it has been mostly associated with prolonged confinement in poorly ventilated spaces in which benzene is employed as a solvent, often in glues. Other solvents, such as toluene and xylene, do not appear to have the same propensity for causing aplastic anemia as benzene.

Benzene can also be found in products used in the home, and in hobbies. Paint removers, and adhesive and cleaning solutions, may contain benzene. The syphoning by mouth of petrol containing benzene has been followed by aplasia. Aplasia has also been reported following exposure to the commercial solvent known as 'Stoddard's solvent', which is used as an all purpose solvent, particularly as a dry cleaning agent, paint thinner and for cleaning machinery.

Aplastic anemia has been attributed to inhalation of vaporized lindane (gamma benzene hexachloride) when used in an insecticide. The vaporizers that volatilize lindane have been widely used in homes, offices, public eating places and industrial workplaces. Lindane is also present in some spray insecticides used in the home and in agricultural work. Aplasia has also been reported following exposure to the insecticides chlordane and chlorophenothane (DDT), and to trinitrotoluene in explosives factories.

APLASTIC ANEMIA AND INFECTIOUS HEPATITIS

Aplastic anemia occurs as a rare, but recognized, complication of infectious hepatitis, usually developing as an apparent idiosyncratic phenomenon after recovery from the hepatitis has taken place. It is most commonly associated with epidemic hepatitis infections

(hepatitis A), and thus occurs more commonly in the younger age group affected by this type of hepatitis virus. Infections with a number of other viruses can cause marrow hypoplasia which is usually transient, although occasional instances of aplastic anemia have been reported.

FANCONI'S ANEMIA

Familial aplastic anemia can occur, and usually the onset is in the first decade of life. There may be associated abnormalities, such as patchy brown cutaneous pigmentation and neurological, renal, or skeletal malformations. Associated biological abnormalities are a diminished capacity for DNA repair and increased random chromosome breakage during mitosis. A resulting aberration involving DNA may serve as an initiating event in the development of aplastic anemia, or of leukemia which occurs more frequently in individuals with decreased DNA repair capability. Although an initial satisfactory response to anabolic agent therapy may occur, the disorder most commonly tends to pursue an inexorable course to fatal pancytopenia.

CLINICAL

Primary

- All ages affected, rarely infants and children involved
- Course is usually chronic, sometimes acute.

Secondary

- All ages affected
- Course is usually acute, sometimes chronic.

SIGNS AND SYMPTOMS

- Anemia, pallor
- Neutropenia, infections
- Thrombocytopenia, bleeding manifestations.

BLOOD PICTURE

- RBC morphology is normal usually
- (Polychromasia, normoblasts and stippling usually not seen)
- Sometimes macrocytosis, mild anisopoikilocytosis
- Hb and PCV are diminished to about 7% and 20% respectively
- Red cell osmotic fragility is normal
- Leukopenia
  - Neutropenia, polymorphs are qualitatively and quantitatively abnormal, show coarse granules
  - Absolute lymphocytopenia but relative lymphocytosis
  - Neutrophil alkaline phosphatase count is increased
- Thrombocytopenia
  - Bleeding time is increased
  - Clot retraction time is increased
  - Coagulation parameters are normal
- Serum iron is increased

- TIBC: saturated
- Radio iron bone marrow uptake is reduced
- Red cell life may be decreased
- Plasma erythropoietin level is raised.

BONE MARROW

Bone marrow may be:
- Aplastic
- Hypoplastic
- Normal or
- Patchily hypercellular

Aspiration

- Blood tap or dry tap
- Is hypoplastic (there is both myeloid and erythroid hypoplasia)
- Fat cells are increased
- 60–100% of the cells are lymphocytes
- Plasma cells and reticulum cells are prominent
- Megakaryocytes diminished in number
- WBCs may show maturation arrest
- Developing granulocytes are abnormal, may show
- Abnormal granulation or
- Vacuolization.
- Bone marrow iron may be normal or increased.

DIAGNOSIS

- Pancytopenia
- Rapid ESR
- No immature cells in peripheral smear
- Bone marrow is aplastic/hypoplastic
  If two consecutive aspirations have been unsuccessful do a bone marrow trephine biopsy
- Differential diagnosis of pernicious anemia—in this, there is pancytopenia without hepato/splenomegaly or lymph node enlargement.
  - Other causes of neutropenia
  - Other causes of thrombocytopenia.

COURSE AND PROGNOSIS

Aplastic anemia is a serious disorder which frequently terminates in death within 6 months. Mortality rates vary in different series from somewhat less than 50%, to as high as 80% in the first year after presentation. It is too early to be certain of the extent to which therapeutic modalities such as allogeneic bone marrow transplantation and administration of antithymocyte globulin will improve survival. Both forms of treatment are associated with some reduction in mortality, but transplantation is currently restricted to patients who have a histocompatible donor, and antithymocyte globulin administration is not always successful or sustained in its effect. It does, however, appear that one or other modality will enable a proportion of patients to survive who would otherwise die from the disorder.

Mortality is usually due to bleeding and/or infection, and most commonly occurs in the first 6 months of presentation. The longer the patient survives, the greater is the chance of improvement in hematological parameters, although partial remission can be followed by subsequent deterioration, and the risk of death remains greater than normal even in long-term survivors. It is not adequately appreciated that aplastic anemia is generally a chronic condition in which the hemopoietic abnormality can persist for years in spite of the withdrawal of causative agents.

In many instances, hematological parameters have continued to deteriorate for a considerable period after exposure to an incriminated drug has ceased. Recovery to complete normality occurs in the minority of cases although the degree of abnormality in other survivors, is frequently compatible with a clinically satisfactory state. Improvement, on rare occasions, can occur over a matter of weeks, but usually occurs slowly, the platelet count is particularly slow to rise, with thrombocytopenia often persisting after the hemoglobin level and leukocyte count have returned to normal. Paroxysmal nocturnal hemoglobinuria sometimes develops during the course of the disorder and acute leukemia in rare instances.

The outcome is difficult to predict in any one individual at presentation, and efforts have been made to identify factors associated with a good or bad prognosis. One analysis suggested that bleeding at presentation, male sex, abrupt onset, low absolute reticulocyte count, more severe thrombocytopenia, more severe neutropenia, and greater depression of hemopoietic cells in the marrow aspirate were associated with a worse prognosis.

PREVENTION

Cautious selection of therapeutic agents. Before prescribing a medicine which is a potential cause of aplastic anemia, the physician should weigh the risk of alternative measures for treating the disease against the risk associated with the drug. No potentially toxic drug should be used if an alternative, effective, non-toxic drug is available. Furthermore, no drug that carries a greater degree of risk than the disease should be used. Many reported cases of fatal aplastic anemia result from administration of potentially toxic drugs to patients with relatively minor complaints which were either self-limiting, or for which an effective, safe, alternative therapy was available. A patient who has developed aplasia following exposure to a particular drug should never receive that drug again, and should be given a warning card to show to all future medical attendants.

MANAGEMENT

The prognosis of the disorder is difficult to predict as it can progress rapidly to a fatal outcome, pursue a relatively indolent course, or recover in rare instances after several weeks. Such variability has made it difficult to establish with controlled trials the benefits of different management modalities. It is, however, clear that substantial improvement may occur after several months of severe pancytopenia, and a basic principle in never to give up attempts to achieve a satisfactory outcome. Management is considered under the following headings:

1. Identification and elimination of exposure to causative agent.
2. Supportive therapy:
   - Prevention and treatment of infections
   - Prevention and treatment of hemorrhage
   - Red cell transfusion for anemia.

3. Measures to accelerate recovery from pancytopenia:
   - Bone marrow transplantation
   - Antithymocyte globulin
   - Anabolic agent administration
   - Corticosteroid administration.

Identification and Elimination of Exposure to Causative Agent

There is no denying the fact that aplastic anemia is precipitated by ingestion or inhalation of certain drugs or chemicals. In such instances, it is logical to terminate the exposure as rapidly as possible in the hope this will lessen the ultimate severity of the disorder. One of the distressing features is that the condition can nonetheless inexorably progress over several months to a fatal outcome after only a brief period of exposure to certain drugs.

Sometimes, it may be difficult to establish the extent and identity of drugs or chemicals to which a patient with aplastic anemia has been exposed in the relevant preceding period of six months. Many patients simply do not reliably recall their medication history, and most do not know the extent to which they have been exposed to chemicals. Some insight into the nature of chemicals, such as benzene, with which individuals may have contact at work or in the home, is therefore valuable in the assessment of potential exposure to such agents. A more reliable drug history can usually be obtained from the patient's medical practitioner, but this will not reveal the ingestion of nonproprietary, so-called 'herbal' medications, which in some countries contain additives such as phenylbutazone.

Supportive Therapy

Prevention and Treatment of Infection

When the patient is at risk from infection because of neutropenia or because of neutopenia or because of glucocorticoid administration, several prophylactic measures are advisable. One is avoidance of areas of microbial contamination such as hospitals, where antibiotic-resistant organisms are common. Reduction of the load of nasal and oral bacteria in patients with severe neutropenia can be achieved with topical antiseptic preparations.

Prophylactic antibiotic administration is not recommended, as it tends to alter the microbial flora to resistant organisms, and such a change is highly undesirable. Isolation of patients with extreme neutropenia in specialized low microbial environments imposes severe limitations on the patient. This may be tolerable during relatively limited phases of extreme neutropenia, such as in remission-induction therapy in acute leukemia or in bone marrow transplantation, but impractical in a condition such as aplastic anemia where little change may occur for prolonged periods. Development of *Candida albicans* infection occurs so commonly in association with administration of high dosages of corticosteroids that concurrent oral administration of a nonabsorbable antifungal agent to suppress the load of such organisms in the mouth gastrointestinal tract should be considered when corticosteroids are given.

Infection represents a serious threat to the severely neutropenic patient with aplastic anemia. The absolute neutrophils count can fall when utilization of neutrophils due to infection exceeds the rate at which neutrophils are formed by bone marrow with diminished productive capacity. Such a development further reduces the defences against infection. Infection can be associated with worsening of the degree of thrombocytopenia, and in already severely thrombocytopenic individuals can precipitate potentially fatal bleeding. Treatment of infection under such circumstances should be prompt and vigorous. Oral

antibiotics may be appropriate for minor episodes of infection in less severely neutropenic subjects, but intravenous broadspectrum antibiotic therapy is indicated when the degree of neutropenia is severe, because of the tendency for the infection to progress rapidly to overwhelming sepsis.

Prevention and Treatment of Hemorrhage

Thromboctyopenia frequently results in bleeding in this condition. While prophylaxis against potentially fatal bleeding with random donor platelet transfusions has been shown to be of value in thrombocytopenic states of relatively limited duration, such as remission-induction therapy for acute leukemia, it has not proved suitable for the more sustained period of thrombocytopenia in aplastic anemia. A major factor is the development of iso antibodies to allogeneic platelets which occurs in response to repeated platelet transfusions over weeks to months, ultimately rendering random donor platelet transfusions ineffective. It has therefore been a common practice to reserve platelet transfusions for treatment of clinically significant bleeding, as 6–8 units of random donor platelets can markedly improve the hemostatic defect due to thrombocytopenia for up to several days. When random donor platelets are ineffective because of isoantibodies, it is possible to obtain effective platelet support with platelets from donors with greater degrees of HLA compatibility, such as siblings, if sibling bone marrow transplantation is not contemplated. Use of blood products from potential bone marrow donors is avoided in the hope of limiting the degree of rejection of subsequently transplanted bone marrow.

A simple but important preventive measure against bleeding is avoidance of the use of nonsteroidal anti-inflammatory drugs. They have dual adverse effects: diminution of the functional capacity of platelets, and production of erosions in the upper gastrointestinal tract which tend to bleed. Menorrhagia can also be a serious problem, and the most practical approach to contain it is the uninterrupted administration of hormonal agents that prevent menstruation.

Red Cell Infusion for Anemia

The reason of transfusion is to raise the hemoglobin level to a level at which anemic symptoms are alleviated, and a comfortable life can be led for a reasonable period before transfusion is again required. Because of the better in vivo survival of red cells from recently taken blood, the use of blood taken within the previous few days is preferable. In the presence of bleeding, appropriately collected blood less than 24 hours old has the advantage of containing functionally effective platelets and coagulation factors; however, selective blood component therapy has generally replaced this practice.

Individual transfusion requirements vary from patient to patient, depending on the severity of the marrow depression and the extent of blood loss due to hemorrhage. Many patients require transfusion at regular intervals, but a few need only an occasional transfusion. It is not necessary to maintain the hemoglobin continually at normal levels.

Many patients are comfortable for 6–8 weeks after the hemoglobin has been raised by transfusion to about 12–14 g/dL, provided that no bleeding occurs. Further transfusion is given when symptoms of anemia again become prominent, usually when the hemoglobin level has fallen to around 7–9 g/dL. Transfusion requirements in women are often greater because of menorrhagia when menstrual activity is not suppressed by hormonal therapy.

In patients with marked bleeding, transfusion requirements are sometimes massive. Transfusions tend to become progressively less effective and are therefore required at increasingly shorter intervals, especially in patients who have received many transfusions.

This is due to the addition of a hemolytic element to the anemia. While this hemolysis sometimes results from the development of isoantibodies, in many cases no such antibodies can be demonstrated. It appears that the spleen plays some role in the increased destruction of transfused red cells, as splenectomy may result in a lowering of transfusion requirements.

An important problem which may develop in patients receiving repeated transfusions is the occurrence of transfusion reactions, which may also lessen the efficacy of the transfusion. Sometimes these are due to the development of red cell isoantibodies which can be detected by careful cross-matching. However, in many such patients, no red cell antibodies can be demonstrated, and it has been shown that the reactions may be due to development of antibodies to leukocytes or platelets. Such reactions can be reduced in severity or avoided by removing the leukocytes and platelets, by taking off the buffy coat when red cell concentrates are prepared by centrifugation, or by freezing the red cells. It is important also to avoid damage to veins which will prevent further transfusion, and thus the cut-down procedure should be avoided. Sometimes, the amount of iron delivered by multiple transfusions is so great that clinical manifestations of hemochromatosis appear.

MEASURES TO ACCELERATIVE RECOVERY FROM PANCYTOPENIA

Bone Marrow Transplantation

Intravenous infusion of HLA-compatible bone marrow in order to engraft effective hemopoietic tissue in the patient's marrow cavity has achieved a considerable degree of success in the treatment of younger patients with severe aplastic anemia. In the rare situation where the donor is an identical twin, the donor tissue is entirely histocompatible and does not give rise to immune mediated attack on the tissues of the recipient, otherwise known as graft-versus-host disease. Bone marrow transplantation now tends to be restricted to patients under the age of 40 years, and is performed by infusion of marrow from an HLA-identical donor whose lymphocytes shows little or no reactivity to recipient lymphocytes in the mixed lymphocyte reaction. Greater degrees of HLA or mixed lymphocyte reaction incompatibility are associated with rejection of the bone marrow graft, or unacceptably severe graft-versus-host disease. The procedure as it is currently performed is one which is applicable to a minority of subjects with aplastic anemia, as fewer than half have an HLA-compatible donor, and complications are of such severity in recipients over the age of 40 years that the procedure tends to be avoided in this age group.

The recipient is given such intense immunosuppressive treatment to prevent graft rejection that it causes extreme marrow suppression. Restoration of effective hemopoiesis by donor marrow infused at this time usually does not take place for 2–3 weeks, and thus specialized facilities and clinical expertise are required to support the patient during the intervening phase of life-threatening pancytopenia. For this reason, better results are obtained in specialized centers.

While most recipients survive the initial phase of pancytopenia, a distressing development is graft-versus-host disease in at least half of the survivors, despite attempted prophylaxis against the condition with immunosuppressive agents such as methotrexate or cyclosporin -A. This is due to immunologically mediated attack by the immune system derived from donor marrow on host cells bearing minor antigenic differences, and it causes potentially fatal injury to tissues such as liver, bowel, and skin. There is commonly suppression of immune responses to pathogens, leading to infections such as pneumonia due to cytomegalovirus, yeasts, fungi, *Pneumocystis carinii* and other organisms. These infections are often fatal. The severity and frequency of such complications are currently

sufficiently great for bone marrow transplantation to be restricted to subjects with very severe aplastic anemia in whom alternative forms of management are associated with a very poor prognosis.

ANTITHYMOCYTE GLOBULIN ADMINISTRATION

Intravenous administration of immunoglobin preparations containing antibody to human thymocytes for approximately one week is associated with improvement in hematological indices in about one-half of the treated subjects.

Improvement occurs relatively slowly, and uncommonly results in sustained normal values, although the degree of change confers considerable clinical benefit. Antithymocyte globulin thus provides potentially beneficial treatment for patients with aplastic anemia in whom bone marrow transplantation cannot be performed because of advanced age or lack of a suitable donor. The antibody currently employed is mostly raised in the horse, and the patient must be carefully monitored during the infusion because of the low but real possibility of developing an anaphylatic response to horse serum components.

What is the best line of action when a histocompatible marrow donor has been identified? Results from allogeneic marrow transplantation are, generally speaking, superior to those obtained with antithymocyte globulin treatment in children and adolescents. A shift towards the reverse situation occurs as the age of the recipient increases further, and results are generally superior with antithymocyte globulin treatment in subjects over the age of 40 years, although allogeneic marrow transplantation still remains an option if the response to antithymocyte globulin is unsatisfactory.

Anabolic Agent Administration

Oxymetholone is currently the most commonly administered anabolic agent for the treatment of aplastic anemia. A variety of androgens have been shown to produce a degree of improvement, and oxymetholone is one orally absorbable form associated with beneficial effects in some patients. Anabolic agents do not appear to decrease mortality in severe aplastic anemia, but are of undoubted benefit in less severely affected cases because they can produce a delayed increase in the cellular elements of the blood. The most dramatic effect is on the hemoglobin level, and may terminate dependence on transfusion. The dosage is usually 100 mg oxymetholone per day, or more, and in some responders, it can be withdrawn successfully after 6–12 months. In other words, the hematological indices may deteriorate after oxymetholone withdrawal and further courses, or maintenance therapy, often effective at lower doses, may be required. Major side-effects include hepatic damage, a problem common to all orally absorbable androgens, and virilization in females.

Corticosteroid Administration

Administration of corticosteroids in high dosages has occasionally been linked with improvement in aplastic anemia. Therapy formerly often consisted of corticosteroids in combination with an anabolic agent, although it is uncertain whether the corticosteroids conferred any benefit. The lack of clearly established benefit in most patients is compounded by the undesirable side-effects of relatively high doses of corticosteroids, such as osteoporotic fractures, glucose intolerance, and increased susceptibility to infection.

PURE RED CELL APLASIA

CONGENITAL OR DIAMOND–BLACKFAN ANEMIA

This type of anemia, described by Diamond and Blackfan in 1938, characteristically develops in infancy, and differs from Fanconi's anemia in that it is not accompanied by impaired production of leucocytes or platelets. It is a chronic disorder, caused by selective depletion of erythroid precursors. Although many explanations have been advanced to account for the lack of erythroblasts, there is currently no consensus as to the pathophysiology of the disorder. Anemia may become extreme, and is not generally associated with other abnormalities, such as thymoma which can accompany acquired pure red cell aplasia. Diamond–Blackfan anemia differs from acquired pure red cell aplasia and aplastic anemia in that the majority of cases respond to glucocorticoid administration, and although maintenance therapy is usually required, the maintenance dosage that sustains effective remission is often surprisingly small. Spontaneous remission may occur during puberty.

ACQUIRED PURE RED CELL APLASIA

The selective depletion of erythroblasts in this condition may involve only the more mature erythroblasts, although most commonly the entire erythroid series is affected. There is characteristically an acquired normochromic normocytic anemia accompanied by a depressed absolute reticulocyte count in the chronic form of the disorder. While the leukocyte and platelet counts are usually normal, depression of leukocytes or platelets occasionally develops at some stage.

Chronic acquired pure red cell aplasia differs from aplastic anemia in that there is very good evidence of an autoimmune etiology in the majority of cases. An association with thymoma is common, although in the minority, and the nature of the relationship with the thymoma is poorly understood. A thymoma can be present many years before pure red cell aplasia develops, or alternatively, may have been excised apparently completely years beforehand. The association with many disorders of an established or presumed autoimmune nature, and the frequently beneficial response to immunosuppressive agents, suggests the condition is commonly due to immune-mediated injury to erythroid precursors.

Surgical excision of an associated thymoma has been reported to produce remissions, although such a result is far from inevitable. Pure red cell aplasis unassociated with thymoma may occasionally respond well to glucocorticoid administration, but sustained benefit in terms of easily maintained or unmaintained remissions are achieved in about two-thirds of cases after courses of immunosuppressive agents, such as corticosteriods plus cyclophosphamide or azathioprine.

DIFFERENTIAL DIAGNOSIS OF PANCYTOPENIA

Aplastic anemia is characterized by pancytopenia in which careful scrutiny of the blood film characteristically fails to reveal erythroid or leukocyte precursors. The erythrocyte sedimentation rate is often markedly elevated, and thus does not serve as a specific index of marrow depression due to infiltration by disorders associated with paraprotein production, such as multiple myeloma or macroglobulinemia. Values in excess of 100 mm/hour are, however, sufficiently elevated to raise the possibility of the presence of the latter conditions.

Clinical examination usually reveals no positive findings other than those of anemia, or of bleeding or infection resulting from thrombocytopenia or neutropenia, respectively. Lymph node enlargement is not characteristically part of the disorder, although regional

nodes draining an infective focus may become moderately enlarged. The spleen and liver are usually impalpable.

Occurrence of one or more of the following signs in a patient with pancytopenia suggests a diagnosis other than aplastic anemia: splenomegaly, hepatomegaly, lymph node enlargement, bone tenderness, immature white or red cells in the peripheral blood, normal erythrocyte sedimentation rate.

The diagnosis is usually established by examination of a satisfactory bone marrow aspirate containing at least a number of marrow particles, or of a bone marrow trephine. Rarely, the picture in the biopsied material is dominated by a focus of active marrow and may lead to an erroneous diagnosis of myelodysplastic syndrome.

Subleukemic leukemia is a more common cause of pancytopenia than aplastic anemia. The leukocyte and platelet counts are depressed on presentation in an appreciable minority of subjects with acute leukemia and the diagnostic problem arises in the subleukemic group when there are very few or no blast cells evident in the blood film. Certain physical signs and, of course, examination of the bone marrow indicate the diagnosis. There may be lymphadenopathy, or enlargement of the liver or spleen, although these features can be present in lymphoma and macroglobulinaemia. Features differentiating this condition from aplastic anemia are summarized in Table 10.4.

Table 10.4: Aplastic anemia vs subleukemic leukemia

	Aplastic anemia	***Subleukemic leukemia***
History	*Relatively recent exposure to chemical agents or drugs in about one-half of cases*	*Occasionally exposure to radiation, alkylating agents or benzene in the past*
Physical examination	Rare	Common
Sternal tenderness Splenomegaly	Uncommon, when present only slight	May be absent at onset, but can develop during course of illness
Lymph node enlargement	No generalized enlargement. Regional nodes draining infective lesions may be enlarged	Sometimes, present and can develop during course of illness
Gum hypertrophy	Absent	Occasionally present
Blood examination		
Red cell morphology	Slight to moderate anisocytosis, often with some macrocytosis. Poikilocytosis of varying degree	Moderate anisocytosis and poikilocytosis usual
Neutrophils	Normal in morphology and granulation	Hypogranulation and Pelgar-Huet-like anomaly may be seen
Immature white and red cells	Usually absent	Absent or present only in small numbers at onset, but appear in the course of the illness. Blast cells predominate
Erythrocyte sedimentation rate	Almost invariably raised	Usually but not invariably raised
Bone marrow examination	Usually hypocellular Occasionally normocellular or hypocellular. Examination at second site usually yields hypercellular specimen	Usually cellular or hypercellular leukaemic tissue. Chromosome abnormalities present in about one-half of cases

Chemotherapy-related bone marrow depression is usually overtly linked to administration of cytotoxic agents or antimetabolities, and thus poses no diagnostic problem. The connection can, however, be obscure when pancytopenia due to bone marrow hypoplasia is caused by inappropriate administration of these agents, in the treatment of nonmalignant conditions, and in situations where toxicity is enhanced by diminished drug elimination. Examples of diminished drug elimination include the retention of methotrexate in subjects with renal impairment, and diminished degradation of azathioprine or 6-mercaptopurine by concurrent administration of the xanthine oxidase inhibitor, allopurinol.

MYELODYSPLASTIC DISORDERS

These disorders can present with pancytopenia. They are a varied group of hematological neoplastic conditions which do not fulfil the criteria of leukemia, although the percentage of blast cells in some forms is above normal in the bone marrow, and blast cells may be present in the blood. Clinical examination can reveal enlargement of the liver or spleen, so that findings of this nature coupled with blasts in the blood film readily distinguish this condition from aplastic anemia. Definition of the type of myelodysplastic syndrome is usually established by bone marrow examination, in which the usual cellular or hypercellular picture differentiates it from aplastic anemia, and the constitution of the cell population usually, but not always, readily differentiates it from acute leukemia.

BONE MARROW INFILTRATION OR REPLACEMENT

Pancytopenia is occasionally present on presentation with lymphoma, either due to bone marrow infiltration in advanced stage disease, or to hypersplenism because of enlargement of the spleen. In most instances the biopsy of an enlarged, readily accessible lymph node establishes the nature of the disorder, although sometimes there is no palpable lymphadenopathy or detectable enlargement of the liver or spleen. The presence of lymphoma may be suggested by the presence of constitutional symptoms, such as fever, night sweats, malaise, and pruritus, or because of radiologically demonstrable mediastinal or hilar lymphadenopathy. It is often difficult to establish a diagnosis of Hodgkin's disease or non-Hodgkin's lymphoma of the nodular or large cell varieties by marrow aspiration when the bone marrow is infilterateded by these disorders. The trephine biopsy is more helpful, as it is far more likely to contain diagnostic material which is difficult to obtain by aspiration. Waldenstrom's macroglobulinaemia is relatively commonly associated with a clinical picture similar to that of advanced stage, well-differentiated non-Hodgkin's lymphoma, and may likewise be accompanied by pancytopenia due to bone marrow infiltration.

MULTIPLE MYELOMA

They present relatively frequently with pancytopenia, although the depression of the blood cell counts is usually only moderate in degree. Distinguishing features are a paraprotein on serum or urine protein electrophoresis, lytic lesions or osteoporosis in bone, and Bence–Jones protein in urine. A histological diagnosis is usually established by examination of the bone marrow, which typically reveals an absolute increase in plasma cells, often with atypical morphological features.

Infiltration of the bone marrow with metastatic cancer is a rare cause of pancytopenia, and is usually accompanied by a leukoerythroblastic blood picture, where a relatively small proportion of the nucleated cells consist of erythroblasts and granulocyte precursors.

Another infiltrative disorder of the bone marrow associated with pancytopenia and leuko-erythroblastic blood film is myelofibrosis. There is usually marked poikilocytosis with tear-shaped red cells and, almost inevitably, splenomegaly in myelofibrosis. The diagnosis in this condition, and in metastatic cancer of the bone, is best established by trephine biopsy of the bone marrow, as aspiration usually fails to obtain particles of bone marrow in myelofibrosis.

Hypersplenism usually causes relatively mild pancytopenia, and should be considered in any subject with splenomegaly. In the absence of complicating factors, the bone marrow contains normal cellular elements which are usually increased in cellularity.

Megaloblastosis due to vitamin B_{12} or folic acid deficiency can, in the extreme case, cause potentially fatal pancytopenia. The blood film usually contains oval macrocytic red cells and hypersegmented neutrophils. Diagnosis is nearly always established by examination of the bone marrow, which is usually hypercellular and contains characteristic megaloblastic erythroid and granulocyte precursors, although on rare occasions the bone marrow is hypocellular.

SYSTEMIC LUPUS ERYTHEMATOSUS

SLE is often accompanied by minor degrees of pancytopenia. Moderate anemia is common and is usually of the type associated with chronic inflammation, although auto-antibody-mediated hemolytic anemia is occasionally the cause. Mild leukopenia is also common, but the leukocyte count rarely falls below 2×10^9/L. Marked depression of the platelet count is more common than severe anemia or leukopenia, and is usually mediated by autoimmune destruction of platelets. Examination of the bone marrow usually reveals relatively cellular tissue without abnormal cells, which excludes aplastic anemia and infiltration by malignant processes. Arthralgia, skin rashes, and other clinical features of systemic lupus erythematosus may be present, and the diagnosis is confirmed by increased DNA-binding capacity or antinuclear antibody levels in the serum.

PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

PNH may present with pancytopenia in which hemolysis may be an inconspicuous feature. The diagnosis of this uncommon condition is indicated by increased susceptibility of red cells to lysis in isotonic sucrose or in the Ham's acid-serum test.

Florid and overwhelming infection can produce pancytopenia, and therefore is not immediately distinguishable on clinical grounds from aplastic anemia in which sepsis has developed as a consequence of inadequate neutrophil production. Bone marrow examination is capable of differentiating between these conditions, as the cellularity is greater in the former situation, even though the more mature cells of the neutrophil series tend to be depleted. Disseminated tuberculosis is a less fulminant cause of pancytopenia of this type. Staining of bone marrow differentiates.

CHAPTER

11

White Blood Cells

PHYSIOLOGY OF WHITE BLOOD CELLS

SITES OF PRODUCTION

Development of granulocyte is normally restricted to the bone marrow after birth. The other important components of the myeloid series are the monocytes, which are also produced predominantly in the bone marrow. Monocytes are related to tissue histiocytes, or macrophages, which are present in particular in the spleen, bone marrow, lungs and liver. The process of development of tissue histiocytes is less clearly defined than that of granulocytes and monocytes. Lymphocytes are also produced in bone marrow, and some undergo subsequent modification in other organs, such as the thymus. The majority of lymphocytes are formed outside the bone marrow in lymph nodes, lymphoid collections lining the gastrointestinal tract, and the spleen.

PRODUCTION AND LIFE SPAN OF WHITE BLOOD CELLS

Normally, mature forms of the white cell series overwhelmingly predominate in the blood, while precursors are located at extravascular sites. The period spent by the polymorphonuclear neutrophil in the bloodstream averages less than one day, and these cells do not re-enter the blood after they have passed out into the tissues. Lymphocytes and monocytes, on the other hand, do reenter the bloodstream, and consequently their traffic through the body is more complex than that of the neutrophil.

Precursors such as myeloblasts and their progeny, the promyelocytes and myelocytes, undergo cell division, and are accordingly classified as the proliferative compartment of the neutrophil series. Such cell replication increases the number of neutrophil precursors which, after reaching the stage of the metamyelocyte, cease proliferation and undergo a sequential series of changes leading to the formation of the mature segmented neutrophil. The latter events are said to take place in the maturation compartment of the neutrophil series.

Segmented and band neutrophils are the only forms which pass in significant numbers into the circulation under normal circumstances. Approximately one-half of these cells are associated sufficiently closely with vessel walls that they are not present in venous blood obtained by venepuncture. The blood neutrophil count therefore represents only about one-half of the total number of neutrophils in the vascular compartment. Intravenous administration of adrenaline causes many of the neutrophils attached to the endothelium to detach and reenter the circulating blood. The increase in neutrophil count under these circumstances is rapid, and provides an index of the extent to which neutrophil count under these circumstances is rapid, and provides an index of the extent to which neutrophils are

present in the attached or 'marginated' state. Corticosteroid administration in amounts equivalent to about 50 mg prednisolone results in a more delayed, but more sustained, rise in the blood neutrophil count. This is due to promotion of the exit of neutrophils from the bone marrow, and accounts for the neutrophil leukocytosis, associated with some immature forms in the blood of patients receiving relatively high doses of corticosteroids.

It is difficult to be certain of the period encompassed by the process of development of the neutrophil from the myeloblast to the time of its entry into the blood, but a currently accepted approximation is about 12 days. The exit of neutrophils from the circulation occurs randomly, and their half-life in the circulation is only of the order of seven hours. The subsequent period spent in the tissues is also considered to be relatively brief. Neutrophils may degenerate following the interaction with microbes, which have invaded tissue, and can also be lost from the body by migration into the respiratory or gastrointestinal tracts. Migration of eosinophils into the respiratory tract may assume significant proportions in allergic states affecting the bronchial mucosa.

The lifespan of lymphocytes is extremely variable, as it is very brief in some lymphocyte subsets, while in other subjects the same cell may recirculate for many years.

METABOLIC AND ENZYMATIC CHARACTERISTICS OF WHITE BLOOD CELLS

Specific biochemical features account for the specialized functions of the various types of leukocytes.

The granules in the neutrophil represent packages of enzymes which are involved in the killing of ingested microbes and the digestion of phagocytosed material. There are at least two separate types of granules on the basis of differences in enzyme content. The so-called primary lightly basophilic staining granules contain, for example, peroxidase and acid hydrolytic enzymes while secondary granules contain alkaline phosphatase and certain other enzymes. Peroxidase is a major neutrophil protein which catalyzes the reaction between the hydrogen peroxide generated in the phagocytosing neutrophil and chloride or iodide ions to chlorinate or iodinate ingested microbes. This process is believed to contribute to the microbicidal function of neutrophils, and deficiency of neutrophil peroxidase has been shown to lessen resistance to mycotic and bacterial infection.

A profuse rapid uptake of oxygen occurs when neutrophils ingest microbes. The oxygen is reduced by NADPH to generate superoxide radicles (O_2^-), which are weakly microbicidal in their own right. Superoxide undergoes enzymatic 'dismutation' to hydrogen peroxide, which alone, or in the presence of peroxidase as described above, exerts powerful microbicidal activity. The ingested microbes are enveloped in vacuoles into which microbicidal agents are discharged (Fig. 11.1).

Alkaline phosphatase can be demonstrated by cytochemical staining of neutrophils in blood smears. Neutrophil alkaline phosphatase activity is derived from the intensity of the microscopically identifiable reaction product of the enzyme within neutrophils, and bears no relation to the activity of alkaline phosphatase in serum. The usual procedure is to examine enzyme activity in 100 consecutive neutrophils, employing a score of 0 for absence of reaction product, 1 for pale diffuse coloration or small areas of granular reaction product, 2 for unevenly distributed granules, 3 for uniformly distributed granules, and 4 where the cytoplasm is entirely filled with dark reaction product. There is an element of subjectivity in assessment, and some variation with technique, so reference must always be made to the normal range of the laboratory. A typical normal range is 10–100 per 100 neutrophils.

Elevated values occur as responses to infection, inflammation, and tissue necrosis due to infarction or trauma. Administration of corticosteriods and oral contraceptive preparations can cause elevated values, and the level also rises during pregnancy to reach a maximum at the time of labor. Abnormally high values can also be present in myeloproliferative disorders such as polycythemia vera and myelofibrosis. High values may also be seen in aplastic anemia and Down syndrome.

Subnormal values are so commonly encountered in chronic myeloid leukemia and paroxysmal nocturnal hemoglobinuria that an abnormally low level is of diagnostic significance. Values are occasionally low in other conditions such as infectious mononucleosis, refractory anemias, hypophosphatemia, and autoimmune states, including autoimmune thrombocytopenia.

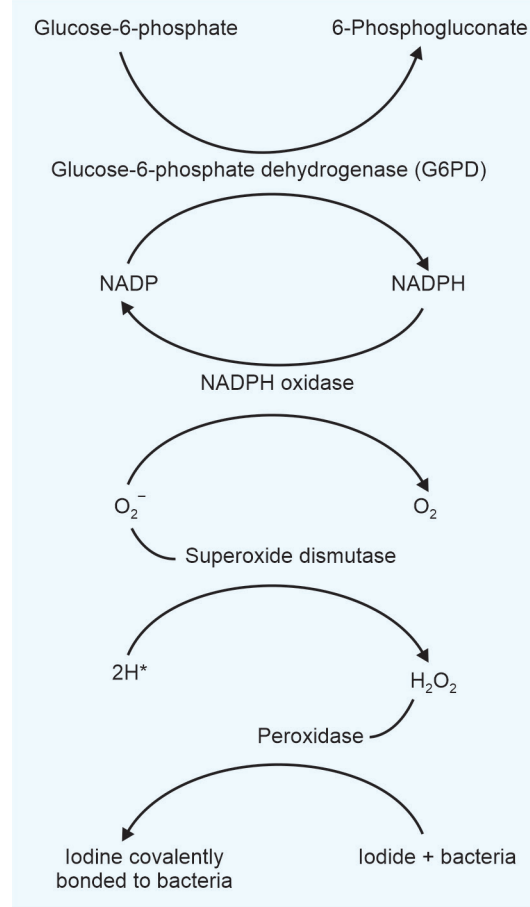


Fig. 11.1: Basic biochemical steps linking the hexose monophosphate shunt and molecular oxygen to oxygen-dependent microbicidal activity in the neutrophil

Lysozyme is a hydrolytic enzyme in the granules of neutrophils and monocytes. It splits the muramic acid of bacterial cell walls, and is consequently also referred to as muramidase. Lysozyme does lyse certain gram-positive cocci, but it fails to make a material contribution to bactericidal activity, and evidently is restricted to aiding the digestion of killed organisms, along with other hydrolytic enzymes packaged in cytoplasmic granules. Lysozyme is released into the plasma, where its concentration reflects the mass of lysozyme containing cells of the body. It is catabolized by the kidney, and is consequently retained in the body in renal failure. In subjects with normal renal function, the serum lysozyme level is of diagnostic value. Conditions associated with increased turnover of cells of the myeloid series, such as infection, are normally associated with moderately elevated levels, although granulomatous states presumably involving large numbers of macrophages, such as sarcoidosis, have been associated in the active phase with very high levels. Very high levels, however, most commonly reflect neoplastic involvement of the myeloid series, and are frequently observed in untreated chronic granulocytic leukemia and acute or chronic myelomonocytic leukemia. The level falls when the neoplastic tissue mass decreases in response to therapy. The opposite extreme of a subnormal level is seen in states associated with depressed numbers of normal myeloid elements in the body.

Enzymes of particular importance to lymphocyte function are also recognized proliferating lymphocytes are endowed with relatively high levels of terminal deoxyribonucleic acid transferase, and this characteristic can be employed as an aid in the

classification of lympoid neoplastic processes. Adenosine deaminase is present in relatively large amounts in T lymphocytes, and is presumably necessary for effective function of a significant subpopulation of cells involved in the immune response, as an inherited deficiency of this enzyme causes as its major clinical consequence a severe combined immunodeficiency state.

FUNCTION OF WHITE BLOOD CELLS

Neutrophils are migratory phagocytic cells. They are attracted by chemotactic factors to sites of microbial invasion and tissue necrosis, where they congregate by passing out of the blood-stream between the endothelial cells of body vessels. Neutrophils are the most important cells involved in the body's defences against micro-organisms, and lack of adequate numbers of neutrophils predisposes to infection, particularly by bacteria. Their ability to locate and ingest microbes is enhanced by attachment of antibody and complement to the microorganism, illustrating the manner in which the various constituents of the body's defences against infection function in a cooperative fashion.

Killing of ingested microbes is mediated by a number of mechanisms, of which the process responsible for the generation of activated oxidative molecules from oxygen is particularly important. Measurement of this mechanism can be performed in the laboratory, using the bonding of radioactive iodide to microbes by neutrophils as an index of their ability to ingest bacteria or yeast, and perform the enzymatic steps culminating in the attachment of iodine to the microbe. Such activity is severely compromised in the potential fatal disorder of neutrophil microbicidal function, chronic granulomatous disease. This is usually an inherited defect in the reaction of molecular oxygen with reduced pyridine nucleotides, and the neutrophils which accumulate at the site of infection ingest but do not readily kill microorganisms (Fig. 11.2).

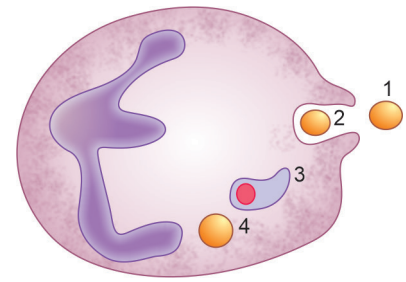


Fig. 11.2: The sequence of events in microbial killing by the neutrophil
1. Complement and antibody coated microbe
2. Particle ingested by neutrophil
3. Enveloped microbe exposed to granule contents plus superoxide and H_2O_2
4. Killed microbe undergoes digestion

Microbial killing is also carried out independently by cationic granule proteins, which presumably react with ingested organisms following the secretion of granule contents into the phagocytic vacuole. A more comprehensive measure of microbicidal function is therefore provided by determination of the effectiveness with which neutrophils kill bacteria or yeast.

Eosinophils also possess phagocytic capacity, but do not normally make a significant contribution to the body's defences against invading bacteria. They congregate in tissues affected by allergic reactions, although their role in the allergic process is not fully understood. Production of eosinophils tends to correlate inversely with corticosteroid levels.

Monocytes are also motile phagocytic cells endowed with microbicidal mechanisms similar to those of neuthrophils, although monocyte phagocytic and microbicidal activity is considerably less effective than that of the neutrophil. Monocytes and macrophages are predominantly located in the extravascular space, and are particularly involved in the phagocytosis and catabolism of necrotic material.

Lymphocytes are motile nonphagocytic cells. There are many subpopulations of lymphocytes which interact with each other and with cells of the monocyte-macrophage series in the maintenance of humoral and cell-mediated immunity.

LEUKOCYTE SURFACE ANTIGENS

Granulocytes and lymphocytes share surface antigens with many tissues, but they also have their own specific surface antigens.

Neutrophil specific antigens promote antibody production following transplacental or transfusion induced immunization of individuals lacking the particular antigen. The major relevance of transplacental immunization is that antibody produced by the mother can cross the placenta and produce neutropenia in the fetus.

HLA antigens on leukocytes are present on other cell types, and represent the major but not sole determinants of histocompatibility of tissues. HLA antigens are grouped into four basic systems, HLAA, HLAB, HLAC, and HLADR (D related). Each system is represented on the cell surface by an antigen which is the product of an allele of the gene for each system. These genes are located in the major histocompatibility complex on chromosome number 6, and each chromosome therefore directs the formation of one antigen of each system. Recognition by the body of cells bearing a foreign HLA pattern underlies the immune-mediated destruction of transplanted or transfused cells that are not histocompatible with those of the recipient.

The HLA genes of each major histocompatibility complex are closely located on the chromosome and are consequently almost always inherited as a single unit. Inheritance follows an autosomal dominant pattern, and this means there is one in four chance that any two siblings will share the same maternal and paternal number 6 chromosomes and consequently be HLA identical, a one in two chance that they will share one parental number 6 chromosome in common, and a one in four chance that they will have no parental number 6 chromosome in common.

The HLA type of an individual is usually determined by studies on peripheral blood lymphocytes. HLA antigens are strongly expressed on these cells, which serve as a conveniently obtainable source of tissue for tissue typing. The degree of incompatibility between individuals is also reflected in the degree to which their lymphocytes undergo a proliferative response when exposed to each other in the mixed lymphocyte reaction. This reaction is largely dictated by differences at the HLADR (D related) gene locus, and serves as a guide to the extent of histocompatibility reactions in transplantation.

NORMAL WHITE CELL VALUES

Table 11.1 illustrates the range in the absolute count of leukocytes encompassed by two standard deviations from the mean, which includes about 95% of normal individuals. White cell counts in infancy and childhood tend to be greater than in adults, with values as high as 25×10^9/L at birth. The count drops over the first seven days of life to about 14×10^9/L. After this time, the absolute neutrophil count falls to approximately he same value as in the adult, although the absolute lymphocyte count remains above adult levels until about 12 years of age.

The leukocyte count normally undergoes a minor degree of diurnal variation because of a slight increase in the afternoon. A number of factors, such as the ingestion of food, physical exercise, and emotional stress, can cause an increase in the count.

Values up to 15×10^9/L are common during pregnancy, and following parturition the count may rise to 20×10^9/L, although it usually returns to normal within a week.

Table 11.1: Total and differential normal white cell values in peripheral venous blood		
		Absolute count × 10^9/L
Total leukocyte count		
Adult		4–11
Infants (full-term, at birth)		10–25
Infants (1 year)		6–18
Childhood (4–7 years)		5–15
Childhood 8–12 years)		4.5–13.5
Differential leukocyte count in adults		
Neutrophils	40–75%	2.0–7.5
Lymphocytes	20–50%	1.5–4.0
Monocytes	2–10%	0.2–0.8
Eosinophils	1–6%	0.04–0.4
Basophils	<1%	0.01–0.1

PATHOLOGICAL VARIATIONS IN WHITE CELL COUNTS

Neutrophilia

Infections

- Pyogenic bacterial, e.g.
  - Staphylococcal
  - Streptococcal
  - Pneumococcal
  - Meningococcal
  - Gonococcal.
- Nonpyogenic, e.g.
  - Acute rheumatic fever
    - Diphtheria
    - Scarlet fever
  - Acute poliomyelitis
    - Cholera
    - Herpes zoster
    - Mycobacterial
    - Fungal
    - Spirochetal
    - Parasitic

Metabolic Disorders

Due to varied causes leading to:

- Acute yellow atrophy of liver
- Uremia
- Diabetes
- Acidosis
- Gout
- Eclampsia.

Neoplasms

- Myeloproliferative disorder
  - Myeloid leukemia
  - Lymphomas
  - Polycythemia vera
  - Myelosclerosis
- Other malignancies
  - Carcinomas (metastatic or otherwise)
  - Sarcomas.

Conditions Causing Cell Necrosis or Destruction

- Acute hemolysis (especially intravascular type)
- Infarctions
- Drug intoxication
  - Nephrotoxins
  - Hepatotoxins

Various drugs/chemicals implicated are:

- Phenacetin
- Digitalis
- Quinine
- Adrenaline
- Organic arsenicals
- Lead
- Mercury
- Carbon monoxide.

Trauma and Hemorrhage

- Hemorrhage
  - Acute hemorrhage (especially internal hemorrhage)
- Trauma
  - Operative
  - Fractures
  - Crush injuries
- Burns.

Cardiac Disorders

Paroxysmal tachycardia

Callagen Disease

- Polyarteritis nodosa
- Acute phase of rheumatoid arthritis
- Dermatomyositis—DLE.

Miscellaneous

- Serum sickness
- Acute anoxia
- Spider venom poisoning
- Histiocytosis-X.

Eosinophilia

Allergic States

- Asthma
- Hay fever
- Exfoliative dermatitis
- Erythema multiforme
- Urticaria
- Food sensitivity
- Angioneurotic edema
- Serum sickness
- Drug allergy.

Parasitic Disease

- Intestinal forms
  - Hookworm
  - Roundworm
  - Tapeworm
- Tissue forms
  - *Toxocara*
  - *Trichina*
  - *Strongyloides*
  - *Echinococcus*
  - Filariasis
  - Malaria.

Skin Disorders

- Pemphigus
- Dermatitis herpetiformis
- Psoriasis
- Scabies
- Prurigo.

Drug Administration

- Liver extracts
- Penicillin
- Streptomycin
- Chlorpromazine

Neoplasms

- Myeloproliferative
  - Eosinophilic leukemia
  - Chronic myeloid leukemia
  - Polycythemia

Others

- Hodgkin's disease
- Multiple myeloma (±)
- Metastatic and necrotic tumors
- Occult abdominal tumor.

Miscellaneous

- Familial eosinophilia
- Eosinophilic granulomatosis (visceral larva migrans)
- Eosinophilic syndrome
- Scarlet fever
- Polyarteritis nodosa
- Tropical eosinophilia
- Pernicious anemia
- Post-splenectomy
- Post-transfusion mononucleosis
- Idiopathic neutropenia.

Lymphocytosis

Acute Infections

- Infectious mononucleosis
- Pertussis lymphocytosis
- Mumps
- Chickenpox
- Rubella
- Infective hepatitis
- Convalescent stage of many acute infections
- Toxoplasmosis
- Influenza.

Chronic Infections

- Brucellosis
- Tuberculosis
- Syphilis (secondary)

Endocrine Disorders

- Thyrotoxicosis
- Adrenal cortical insufficiency
- Hypopituitarism
- Myasthenia gravis.

Neoplasms

- Non-Hodgkin's lymphomas
- Chronic lymphatic leukemia
- Lymphosarcoma
- Multiple myeloma.

Monocytosis

Infections

- Bacterial
  - Brucellosis
  - Tuberculosis
  - Subacute bacterial endocarditis
  - Typhoid fever
  - Recovery stage of an acute infection

- Rickettsial
  - Rocky mountain spotted fever
  - Typhus
- Protozoan
  - Malaria
  - Kala-azar
  - Trypanosomiasis
  - Oriental sore
- Viral
  - Infectious mononucleosis.

Neoplasms

- Monocytic leukemia
- Hodgkin's and other lymphomas
- Myeloproliferative disorders
- Multiple myeloma
- Carcinomatosis

Collagen Diseases

- Rheumatoid arthritis.
- SLE

Miscellaneous

- Chronic ulcerative colitis
- Regional enteritis
- Sarcoidosis
- Lipid storage diseases
- Hemolytic anemia
- Hypochromic anemia
- Recovery from agranulocytosis.

Basophilia

- Chronic myeloid leukemia
- Myelosclerosis
- Polycythemia vera
- Hypersensitivity states
- Myxedema
- Iron-deficiency anemia (some cases)
- Hemolytic and toxic anemias of long-standing
- Preleukemia (some cases)

Basophilopenia

- Neutrophil leukocytosis or leukemoid reaction associated with
  - Infection
  - Neoplasia
  - Tissue necrosis
  - Acute anemia
- Allergic conditions

- Hyperthyroidism
- Myocardial infarction
- Cushing's syndrome
- Following prolonged corticosteroid therapy.

Eosinopenia

Drug/Hormone Therapy

- Adrenocortical steroids
- Adrenaline
- Ephedrine
- Insulin.

Response to Stress

- Acute infections
- Traumatic shock
- Surgical operations
- Severe exercise
- Burns
- Acute emotional stress
- Exposure to cold

Endocrine Diseases

- Cushing's disease
- Acromegaly

Miscellaneous

- Aplastic anemia
- DLE.

Lymphopenia

- Severe pancytopenia
- Congestive heart failure
- Adrenocorticosteroid therapy (transient).
- Advanced Hodgkin's disease
- AIDS (selective T lymphocytes depletion)

BONE MARROW PLASMACYTOSIS

Acute Infections

- Rubella
- Rubeola
- Varicella
- Infective hepatitis
- Infectious mononucleosis
- Scarlet fever.

Chronic Infections

- Tuberculosis
- Syphilis
- Fungal.

Allergic States

- Serum sickness
- Drug reactions.

Collagen-vascular Disorders

- Acute rheumatic fever
- Rheumatoid arthritis
- SLE.

Neoplasms

- Disseminated carcinoma
- Hodgkin's disease
- Multiple myeloma.

Other

Cirrhosis of liver.

CAUSES OF LEUKOERYTHROBLASTIC CHANGE

- Metastatic carcinoma in the marrow
- Myelofibrosis
- Leukemia
- Multiple myeloma
- Hodgkin's disease
- Non-Hodgkin's lymphoma and histiocytic tumors
- Miliary tuberculosis
- Severe megaloblastic anemia
- Severe hemolysis, particularly in the young
- Osteopetrosis (Albers–Schonberg disease)

NEUTROPHIL ALKALINE PHOSPHATASE

This enzyme is present in abundance in mature/functional neutrophils. This enzyme is stained for and then scored (depending upon the quantity seen in the neutrophils).

Neutrophil Alkaline Phosphatase may be Raised in

- Bacterial infections
- Myocardial infarction
- Trauma
- Diabetic acidosis
- Polycythemia vera
- Myelosclerosis
- Following corticosteroid therapy
- During pregnancy
- Use of hormonal contraceptives

Lowered in

- Chronic myeloid leukemia
- Paroxysmal nocturnal
- Hemoglobinuria

} always

- Idiopathic thrombocytopenic purpura
- Infectious mononucleosis
- Pernicious anemia relapse
- Collagen diseases

} sometimes

- Aplastic and refractory anemias
- Hypophosphatemia

} sometimes

The enzyme muramidase is found in neutrophils, monocytes and monoblasts, serum muramidase estimation is a good indicator of granulocyte turnover.

NEUTROPENIA AND AGRANULOCYTOSIS

Neutropenia is said to be present when the concentration of neutrophils in the blood is below the lower level of normal, i.e. 2×10^9/L. The term agranulocytosis is not as clearly defined. Literally, it means absence of granulocytes from the peripheral blood. Schultz in 1922 introduced the term to describe a clinical syndrome characterized by complete or 'almost complete' absence of neutrophils in the peripheral blood, with severe constitutional symptoms and marked necrotic ulceration, especially of the mouth. The term agranulocytic angina is often used to describe the association of ulceration of the throat with severe neutropenia; however, as infection is not necessarily confined to the mouth, the term agranulocytosis with infection is to be preferred. Many clinicians use the term agranulocytosis to describe severe neutropenia, irrespective of whether infection is present.

NEUTROPENIA AND THE ATTENDANT RISK OF INFECTION

The neutrophil is an essential component of the body's defences against infection, and an inadequate supply of neutrophils profoundly enchances microbial peneration of the tissues. There are factors other than the degree of neutropenia that influence susceptibility to infection, as the neutrophil count does not specifically indicate the number of marginated neutrophils in the vascular compartment, nor the capability of neutrophil precursors in bone marrow to increase production in response to infection. Risk of infection is in fact not appreciably increased at neutrophil counts in the range of $1–2 \times 10^9$/L in the absence of additional complicating factors. It should also be understood that a small percentage of the normal population has counts more than two standard deviations below the mean, and thus have counts below the conventionally employed lower limit of normal. Certain racial groups also have a lower limit of normal than caucasians. Failure to appreciate these factors can result in unnecessary anxiety and investigation in individuals with marginally low neutrophil counts in whom there are no other grounds for suspecting the presence of any clinically significant disorder. The incidence and severity of infection become progressively greater as the neutrophil count falls below 0.5×10^9/L, although in some conditions, such as chronic idiopathic neutropenia, extremely low counts are associated with very much less morbidity and mortality than in individuals undergoing chemotherapy for acute leukemia, for instance.

CAUSE OF NEUTROPENIA

Many conditions are associated with neutropenia, as discussed below. They range from disorders that affect hemopoietic tissue in general to the neutrophil series in particular, and are discussed in detail in the relevant chapters.

Replacement of normal hemopoietic tissue in bone marrow, especially by hemopoietic or lymphocytic neoplastic states, e.g. acute leukemia, myelofibrosis, lymphoma, multiple myeloma, myelodysplasia.

Infections

Especially certain bacterial infections such as typhoid, and early stages of many viral infections such as infectious hepatitis.

Overwhelming Sepsis in which Consumption of Neutrophils Exceed Production

Megaloblastosis

- Cytotoxic therapy
- Felty's syndrome
- Hypersplenism
- Drug or virally induced transient severe neutropenia (agranulocytosis)
- Systemic lupus erythematosus
- Aplastic anemia
- Neonatal isoimmune neutropenia
- Chronic idiopathic neutropenia
- Pseudoneutropenia due to abnormally high proportion of neutrophils in the marginated state.
- Transient post-hemodialysis neutropenia
- Spurious neutropenia in healthy individuals where the count is less than the conventionally stated lower limit of normal.
- Cyclic neutropenia
- Miscellaneous

Miscellaneous

- SLE
- Chronic idiopathic neutropenia
- Anaphylaxis
- Myelokathexis
- Antileukocyte antibodies
- Immunodeficiencies
- Pancreatic exocrine deficiency
- Infantile genetic agranulocytosis
- Cyclic neutropenia (familial and sporadic)
- Congenital aleukia
- Chronic granulocytopenia of childhood
- Lazy leukocyte syndrome

  Symptomatic neutropenia occurs usually in—aplastic anemia, drug Induced neutropenia. hypersplenic and idiopathic neutropenia and acute leukemia.

CLINICAL

- Neutropenia causes lowered resistance to infections, bacterial especially.
- Symptoms in individual patients may appear at different counts.
- Clinical manifestations usually appear when the neutrophil count goes <500 cells/mm^3.

Manifestations in Acute Neutropenia may be

- Constitutional symptoms
- Infective lesions.

Constitutional Symptoms

Constitutional symptoms are those associated with severe infections, i.e. fever leading on to prostration ending at pneumonia.

Infective Lesions

Infective lesions due to invasion of mucous membranes and skin. Ulcerations may appear in skin, mouth, pharynx and GIT.

In Subacute Cases

In subacute cases, the features are the same but less pronounced.

In Chronic Neutropenia

Constitutional symptoms are fatigue, weakness and lassitude because of infection or anemia. Infections are prolonged, severe and multiple. Skin, upper respiratory tract, middle ear and urinary tract infections are common.

DRUG-INDUCED NEUTROPENIA

1. Drugs that cause aplastic anemia
2. Drugs that induced selective neutropenia

Antipyretic, analgesics
- Amidopyrin

Antithyroid drugs
- Thiouracil, methimazole, carbimazole

Antihistamines
- Promethazine, chlorpheniramine, mepyramine, etc.

Tranquillizers
- Chlorpromazine, meprobamate, imipramine, amitryptiline, etc.

Anti-bacterials
- Tetracycline, streptomycin, ristocetin, salazopyrin, sodium, methicillin, etc.

Anti-coagulants
- Phenindione, dicoumarol

Antituberculars
- Isoniazid, PAS, thiacetazone

Antimalarials
- Pamaquin, amodiaquin

Diuretics
- Chlorothlazide, mercurials

Miscellaneous
- Procainamide, penicillamine, metronidazole, etc.

Mechanisms of Neutropenia Induction

1. Direct toxic effect on the bone marrow
2. Destruction of neutrophils in the peripheral blood (both may contribute in some cases).

Clinical Features

Already discussed elsewhere.

Blood Picture

- Neutropenia with no anemia or thrombocytopenia
- In some cases there may be lymphopenia and monocytopenia also.
- Neutrophils may show toxic and degenerative changes
- ESR is usually raised.

Bone Marrow

- Absence of granulocytic precursors with normal erythropoiesis and a normal number of megakaryocytes (sometimes depleted)
- Toxic granulation in developing granulocytes
- Granulocytic hyperplasia implies recovery.

Diagnosis

History of drug ingestion and other findings.

Treatment

- Withdrawal of offending drug
- Antibiotics, after culture and sensitivity
- Corticosteroid administration
- Isolation of patient with barrier nursing
- Granulocyte transfusion
- General supportive and symptomatic therapy, e.g. liquid diet if swallowing is painful.
- (20% patients still die despite measures taken).

CHRONIC IDIOPATHIC NEUTROPENIA (AGRANULOCYTOSIS)

Unknown etiology, persistent neutropenia, recurrent chronic infections. Bone marrow findings are variable leukoagglutinins are responsible.

Clinical

- All ages but more often seen in young women
- Present with mouth ulcers, lassitude and weakness may be present
- Regional lymphadenopathy.

Blood Picture

- Neutrophil count ranges between 500 and 2,000 cells/mm^3.
- Absolute or relative lymphocytosis with or without a moderate monocytosis
- ESR is often raised.

Diagnosis

Persistent neutropenia without any obvious cause.

Differential Diagnosis

1. Drug/chemical induced neutropenia
2. Subleukemic leukemia
3. Malignant lymphoma in elderly patients it runs a chronic course. Spontaneous recovery may or may not occur, death may occur because of pneumonia and septicemia (Table 11.2).

Table 11.2: Neutropenia–differential diagnosis–features

	Agranulocytosis due to drug idiosyncrasy	*Aplastic anemia*	*Acute leukemia*	*Idiopathic neutropenia*	*Hypersplensim*
Frequency	Occasional	Uncommon	Relatively common	Rare	Relatively common
History	Concurrent or very recent drug ingestion	Drug ingestion or exposure to chemicals in about one-half several weeks to several months previously	Rarely exposure to benzene, alkylating agents, or radiation in the past		Features of conditions resulting in splenomegaly
Lymph node enlargement	Absent except in areas draining infected lesions	Absent except in areas draining infected lesions	Sometimes present	Absent except in areas draining infected lesions	Sometimes present as a feature of an underlying disorder
Splenomegaly	Absent	Absent	Sometimes present	Sometimes slightly enlarged	Present
Sternal tenderness	Absent	Absent	Sometimes present	Absent	Absent
Anemia	Usually absent	Present	Present	Usually absent	Common
Very immature white cells	Usually absent	Absent	Common	Absent	Absent
Thrombo-cytopenia	Usually absent	Present	Present	Absent	Common
Bone marrow	Granulocytic series hypoplastic. Erythroid series and megakaryocytes usually normal	Hypocellularity of granulocytic, erythroid and megakaryocytic series	Leukemic or myelodysplastic infiltration	Depletion of some mature granulocytic series. Erythroid series and megakaryo-cytes normal	Usually hyper- or normocellular

Treatment

For prevention and treatment of infection, corti-costeroids may be used if the need arises.

FELTY'S SYNDROME

The association of chronic neutropenia with splenomegaly and rheumatoid arthritis is called Felty's syndrome. Other abnormalities may occur in patients with this condition, including weight loss, pigmentation of the skin, and ulceration of the lower parts of the legs. Felty's syndrome tends to occur in middle age or the elderly, and rheumatoid arthritis usually occurs before the development of neutropenia by many years, although the inflammatory activity of the arthritis may become quiescent before the onset of the neutropenia. Sometimes, the detection of a palpable spleen precedes the neutropenia, and sometimes the reverse occurs.

INFECTIOUS MONONUCLEOSIS

Etiology

- Epstein–Barr virus (the causative agent) (some cases due to cytomegalovirus or toxoplasmosis)
- It may occur sporadically or epidemically
- Glandular fever syndrome, in addition, encampasses toxoplasma and cytomegalovirus infections.

Clinically

Occurs worldwide, affects more commonly older children and young adults, males are affected more often. Incubation period is 5–7 weeks (Table 11.3).

Table 11.3: Clinical presenting manifestations and associated complications of infectious mononucleosis

Common
• Constitutional symptoms of malaise, anorexia, nausea, fatigue
• Fever
• Pharyngitis
• Headache
• Lymphadenopathy
• Splenomegaly
Intermediate frequency
• Myalgia
• Periorbital swelling
• Hepatomegaly or hepatic tenderness
• Mild thrombocytopenia
Uncommon but important
• Skin rash
• Conjunctivitis
• Purpura or bleeding due to severe thrombocytopenia
• Pneumonitis
• Arthralgia
Rare
• Splenic rupture
• Hemolysis
• Neurological problems

Blood Picture

- There is both absolute and relative lymphocytosis, large number of them abnormal
- Hemoglobin value and platelet count are normal
- Initial leukopenia due to reduction in number of neutrophils, the neutrophil alkaline phosphatase count is often low
- Lymphocytosis is maximum at about the tenth day
- Lymphocytes, decribed by downey and mckinely are seen
  - Type I—monocytoid lymphocytes (commonest)
  - Type II—plasmacytoid lymphocytes type III—blastoid lymphocytes
- ESR is raised in 50 percent of cases.
- Wasserman reaction may be positive in 3–10% cases.

Paul–Bunnell Test for Heterophile Antibody

Based upon the presence of antisheep red cell hemagglutinins in unusually high titers in the sera of these patients. It is variable in about 80–90% of cases. It remains positive for a variable period of time. In addition to the said antibodies at least 2 other types of agglutinins for sheep red cells occur in human serum. They are:

a. An antibody present in low titres in normal persons and in malignant lymphomas. This antibody is absorbed by guineapig kidney but not by ox cells.
b. An antibody occurring following the injection of horse serum and in serum sickness. This is absorbed by both guineapig kidney and ox cells.

The antibody of infectious monoucleosis is not absorbed by guineapig kidney but is absorbed by ox cells.

Differential Diagnosis

Disorders of Similar Clinical Onset

i. Acute infections and infectious disorders: Malaise, fever and headache, e.g. influenza, upper respiratory tract infections, brucellosis, typhoid fever, bacterial endocarditis, tuberculosis and septicemia.
ii. Disorders causing membranous pharyngitis: Acute leukemia, acute tonsillitis, Vincent's angina, diptheria and agranulocytic angina.
iii. Cases with a rash: Rubella, serum sickness.
iv. Infective hepatitis.
v. Toxoplasmosis.
vi. Acute abdominal disorders.
vii. Disorders of the nervous system: Bengin lymphocytic meningitis, encephalitis, polyneuritis.
viii. Cat-scratch disease: Lymph node histology is characteristic besides a history of cat-scratch.

Disorders with a Similar Blood Picture

i. Cytomegalovirus mononucleosis.
ii. Post-transfusion (post-perfusion) mononucleosis.
iii. Toxoplasmosis
iv. Acute lymphatic leukemia
v. Acute infectious lymphocytosis
vi. Others
   - Infective hepatitis
   - Serum sickness
   - Bacterial endocarditis
   - Hodgkin's disease
   - Brucellosis

Course: Usually self-limiting within 2 weeks, convalescence of similar period. Sometimes may be prolonged for months and rarely for years.

DISORDERS OF PHAGOCYTIC LEUKOCYTES CHARACTERIZED BY MORPHOLOGIC CHANGES

These include:

- Pelger–Huet anomaly
- Alder–Reilly anomaly,

- May–Hegglin anomaly
- Chediak–Steinbrinck–Higashi anomaly
- Mucopolysacchardoses VI and VII
- Other causes of lymphocyte vacuolation
- Abnormal specific granule formation
- Familial vacuolisation of leukocytes (Jordan's anomaly).

Pelger–Huet Anomaly (Fig. 11.3)

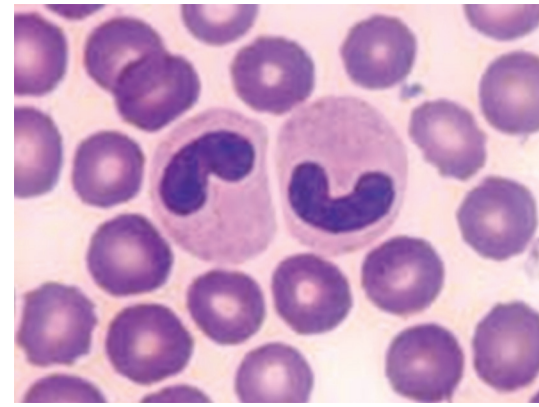


Fig. 11.3: Pelger–Huet cell, peripheral smear

- It is inherited as a non-sex linked, dominant trait.
- It is characterized by distinctive shapes of the nuclei of leukocytes, by a reduced number of nuclear segments (best seen in neutrophils) and by coarseness of the chromatin) of the nuclei of neutrophils. lymphocytes and monocytes. Rod-like, dumbbell, peanut shaped and spectacle-like (pince-nez) nuclei with smooth, round, or oval individual lobes contrast with the irregular lobes seen in normal neutrophils. It should be differentiated from shift to the left that occurs in infection. Pelger–Huet cells appear to be normal in function.

Pseudo or Acquired Pelger–Huet Anomaly

Cells with morphologic changes like those described above have been noted occasionally in association with myxoedema, acute enteritis, agranulocytosis multiple myeloma, malaria, leukemoid reactions secondary to metastases to the marrow, drug sensitivity, or chronic lymphocytic leukemia.

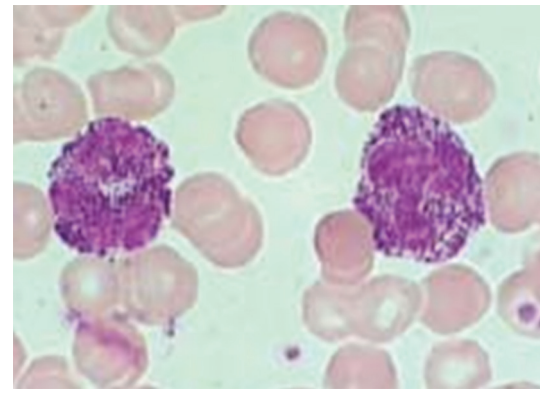


Fig. 11.4: Alder–Reilly anomaly, peripheral smear

Alder–Reilly Anomaly (Fig. 11.4)

Inherited as a recessive trait, is characterized by the presence of larger than normal azurophil and basophil granules (Alder Reilly bodies). These are associated more often in individuals having bone or cartilage abnormalities.

May–Hegglin Anomaly (Fig. 11.5)

This is a rare, dominantly inherited disorder characterized by large (2–5 μm), well defined, basophilic and pyronionphilic inclusions in granulocytes and accompanied by variable thrombocytopenia and giant platelets containing a few granules. These patients may suffer from bleeding diathesis.

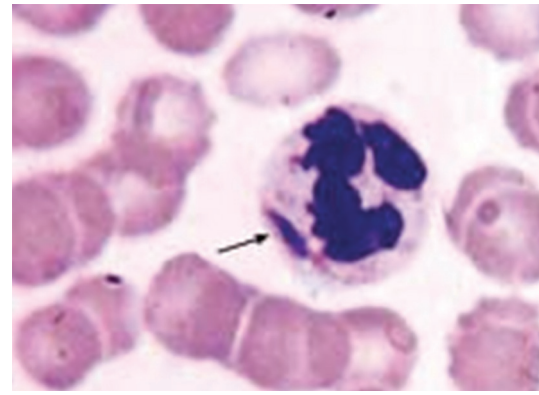


Fig. 11.5: May–Hegglin anomaly, peripheral smear (see inclusion within the neutrophil)

Chediak–Steinbrinck–Higashi Anomaly (Fig. 11.6)

This is characterized by partial cutaneous and ocular albinism, increased susceptibility to pyogenic infections and the presence of large lysosome like organelles, in most granule containing cells and a bleeding tendency.

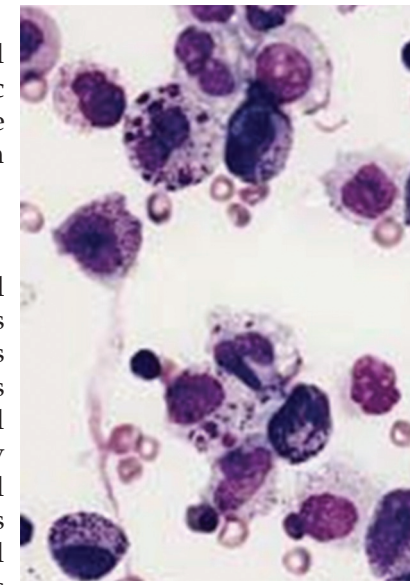


Fig. 11.6: Smear as seen in Chediak–Steinbrinck–Higashi anomaly

Mucopolysaccharidoses VI and VII (Fig. 11.7)

Abnormal granulation of blood granulocytes and monocytes together with lymphocyte vacuolation is found in the Maroteaux–Lamy syndrome—which is also known as abnormality may also be seen in patients with mucopolysaccharidosis VIII. These lysosomal storage disorders are caused by inherited deficiency of enzymes concerned in the breakdown of acid mucopolysaccharides. Storage-related abnormalities of connective tissue, the heart, the bony skeleton and the central nervous system produce clinical disabilities similar to, but milder than, those found in Hurler's syndrome.

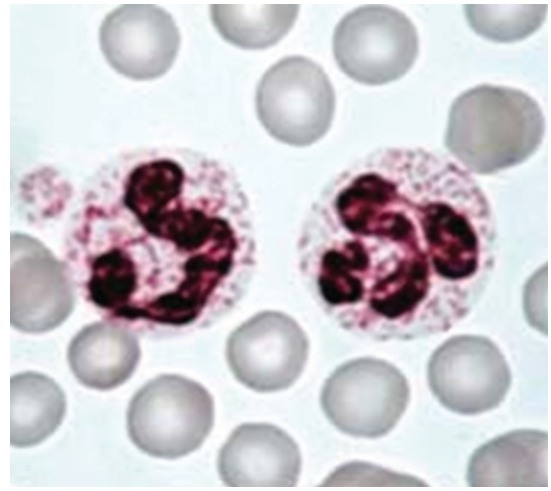


Fig. 11.7: Mucopolysaccharidosis, peripheral smear

Other Causes of Lymphocyte Vacuolation

Similar lymphocyte vacuoles may be found in rare patients with inherited defects of enzymes which are involved in the catabolism of oligosaccharide component of glycoproteins, for example, mannosidosis, and in rare Spielmeyer–Vogt syndrome.

CHAPTER

12

Leukemias

Leukemias are malignancies of hematogenous/hemopoietic cells or tissues. They are diseases in which abnormal proliferation of hemopoietic cells cause progressively increasing infiltration of the bone marrow, although in certain forms the lymphatic tissues are particularly affected. The process of differentiation in leukemic cells is often abnormal, and this commonly results in an immature morphological appearance. Leukemic cells are usually present in the peripheral blood, where the count ranges from very low to very high values. These cells very commonly have myeloid or lymphocytic characteristics, but occasionally cells with features of erythroid precursors or megakaryocytes are part of the disease process.

The rate of progression varies considerably in different types of leukemia, but death is the usual outcome in untreated disease as a result of compromised production of mature blood cells. A significant proportion of subjects with acute leukemia have remissions induced by treatment, and a substantial proportion appear to be cured in certain categories of acute leukemia, especially acute lymphoblastic. A majority of patients with leukemia still die as a result of the disease, although the extent to which survival is prolonged by treatment has increased considerably over the past two decades. Therapeutic measures can improve the quality of life in the presence of persisting disease, and significantly prolong life expectancy when remission of the disease is achieved.

Leukemia accounts for about 4% of all deaths from malignant disease, although the proportion is greater in childhood.

ETIOLOGY

Leukemia is generally considered to be a neoplastic disorder originating in a hemopoietic cell which has undergone an intrinsic change, causing it to escape from the normal restraints imposed on proliferative activity. Leukemic cell populations can consist of cells of one or several different pathways of differentiation.

A critical step in leukemogenesis appears to be alteration of the structure of DNA in the nucleus of the cell in which the disease is initiated, but the precise nature of the underlying changes at the molecular level in the development of human leukemia remains to be established. Many contributory factors have been incriminated in the development of neoplastic change, and include inherited predisposition, effects of viruses, and effects of radiation and chemicals.

MOLECULAR BIOLOGY OF LEUKEMOGENESIS

There is evidence that leukemia in humans can be associated with inappropriate expression of certain genes which appear to be involved in regulating basic steps in cell proliferation.

The normal counterparts of these genes are referred to as proto-oncogenes, and in most instances the precise role played by the gene products in cell behavior remains to be clarified, although they can, for example, correspond to a receptor for a growth promoting factor, or enzymes involved in pathways that convey information to the nucleus that such receptors have been activated. The name for this group of genes is derived from their capacity to undergo constitutive change to oncogenes, characterized by inappropriate expression of the gene in conjunction with neoplastic behavior of the cell.

The genetic code for an oncogene is carried in the RNA sequence of certain RNA retroviruses, and is converted into a DNA copy by viral reverse transcriptase after the virus has infected a susceptible strain of animal or bird. The segment of DNA is incorporated permanently into the DNA of the nucleus, and can result in the development of neoplastic behavior. There is no evidence that retrovirus infection is a common cause of neoplasia in humans, although it does cause a relatively uncommon variant of T lymphocytic leukemia. There are, however, mechanisms that serve as means for activation of oncogenes, other than introduction of an oncogene into nuclear DNA as the reverse transcript of part of the genome of an infecting retrovirus. These mechanisms include changes as small as point mutations in the proto-oncogene, formation of multiple copies of the proto-oncogene, or gene amplification, and of particular relevance to human leukemia, rearrangement of DNA by translocations of chromosomal material.

An example of conversion of a protooncogene to an oncogene by chromosome translation is the activation of the Abelson cellular proto-oncogene (c-abl) by the translocation which results in the formation of the *Philadelphia chromosome*, recognized many years earlier as an association with chronic myeloid leukemia. In this reciprocal translocation between two chromosomes, the c-abl on part of chormosome 9 is transferred into the breakpoint cluster region (BCR) of chromosome 22, as shown in Fig. 12.1. At the same time, part of the long arm of chromosome 22 is transferred onto the long arm of chromosome 9. It appears that segments of c-abl DNA are joined to a DNA sequence in chromosome 22 which results in ongoing production of an Abelson protein with altered structure and function. It is uncertain how this is related to the specific features of chronic myeloid leukemia, and there appear to be other factors involved in the type of malignant change associated with inappropriate activation of the c-abl proto-oncogene. The Philadelphia chromosome may, for example, be the sole chromosomal abnormality in a form of acute lymphoblastic leukemia, indicating there are other influence affecting the characteristics of the neoplastic process. Tumors produced in animals by retroviral introduction of this oncogene are also usually different in character from chronic myeloid leukemia.

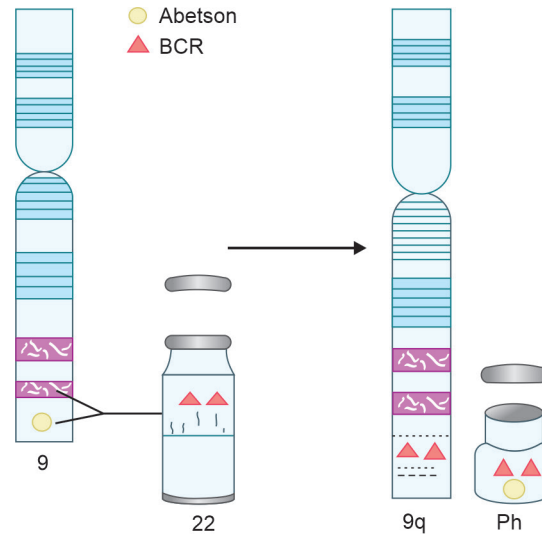


Fig. 12.1: Formation of the Philadelphia (Ph) chromosome by reciprocal translocation of parts of the long arms of chromosomes 9 and 22. The Ph chromosome and the abnormal chromosome 9q⁺persist and are replicated along with the other chromosomes in proliferating chronic myeloid leukemia cells. The translocation shifts the Abelson gene into the BCR site on chromosome 22, and this alters the regulation of its expression

It is commonly believed that inappropriate activation of other genes involved in critical steps in cellular proliferation may be promoted by other types of chromosome translocation, and result in the genesis of other forms of leukemia. Another commonly held view is that permanent changes in nuclear DNA occur in a stepwise manner, and that the outcome of the accumulation of abnormalities is malignant behavior.

ABNORMALITIES OF THE CHROMOSOMES

These abnormalities are very common in leukemia, and occur in all major categories of the acute and chronic forms of the disorder. Usually, the abnormality is maintained in a consistent manner in a particular case, in keeping with a defect that is replicated with a high degree of fidelity in the clone of cells arising from the cell in which malignant behavior first developed. Sometimes, additional changes occur at the time the characteristics of the leukemia alter, suggesting the abnormalities of the chromosomes are directly involved in abnormal cellular behavior.

Not all cases of leukemia have chromosome abnormalities detectable at the microscopic level with currently available techniques. The introduction of banding and specialized staining methods markedly enhanced the precision with which abnormalities in chromosomes could be identified, and it is now possible to recognize translocations between chromosomes which would not have been previously possible. Other types of abnormalities involving chromosomes includes addition or deletion of chromosomes, deletion of parts of chromosomes, and formation of chromosomes that are not identifiable as originating from any particular normal chromosome. More than half of all cases of acute leukemia have chromosome abnormalities, and it is argued that improvement in techniques for delineating chromosome structure are likely to reveal an even high incidence of deletions, additions, and translocations.

A number of correlations exist between laboratory features or clinical behavior of leukemic processes and specific chromosome abnormalities, such as the translocation between the long arms of chromosomes 15 and 17 in acute promyelocytic leukemia. Other instances are the correlations between specific chromosome abnormalities and disorders with relatively good, or relatively bad, prognosis. Associations between particular types of chromosome abnormality and specific properties of neoplastic processes incriminate chromosome abnormalities as playing an important role in the genesis of specific characteristics of neoplastic activity although it remains to be clarified whether chromosome abnormalities could be secondary consequences of another event which is responsible for malignant behavior. One currently held hypothesis is that changes may occur in DNA which produce a clone of cells predisposed to undergo further change, which in turn results in emergence of an overtly leukemic cell clone containing particular chromosome abnormalities.

RADIATION

There is convincing evidence that ionizing radiation, especially X-irradiation, is leukemogenic, and that the incidence of leukemia increases with the cumulative dose received. An increase in the incidence of leukemia was first noted in irradiated survivors of the atom bomb blast three years after the event, and the incidence peaked after 6–7 years. Patients with ankylosing spondylitis treated with radiotherapy also experienced a dose-related increase in incidence of leukemia, reaching a maximum incidence of at least several times the average expectancy about six years after irradiation. Acute leukemia and chronic myeloid leukemia occurred in both irradiated groups. Patients treated with radiotherapy

for lymphoma subsequently develop leukemic or myelodysplastic disorders, but more commonly when cytotoxic chemotherapy is also administered. The incidence of secondary hematological malignancy is relatively high in this group, affecting about 3% of long-term survivors. Concern is also expressed over an increased incidence of leukemia in children who are exposed to diagnostic X-ray *in utero*. The mechanism linking irradiation with development of leukemia upto many years later is unclear, but it is perhaps relevant that irradiation produces chromosome abnormalities, although these take the form of random breaks rather than specific abnormalities in chromosome structure.

CHEMICALS

Leukemia is more common in people who have been exposed for substantial periods to benzene vapor, and the risk of developing leukemia has been suggested to increase with the extent of cumulative exposure. Leukemia also occurs more commonly in subjects who have taken cytotoxic agents which in the case of radiotheraphy, produce an increase in the incidence of chromosome breakage. The drugs most commonly implicated are alkylating agents, such as chlorambucil, melphalan, procarbazine and nitrosoureas. This problem thus affects patients who have received these drugs for treatment, for example, of multiple myeloma, polycythemia vera, lymphoma, ovarian cancer, and breast cancer.

VIRUSES

Various forms of leukemia in animals and birds serve as models for the generation of leukemia as a result of infection by retroviruses. Leukemogenic viruses carry their genetic information in RNA, and after infecting a cell, produce a DNA copy by means of the enzyme reverse transcriptase, which they contain. The DNA copy, or provirus, is incorporated into the DNA of the nucleus, where it is replicated by the cell along with the host nuclear DNA. Malignant transformation is regularly induced when the viral genome contains an oncogene. Oncogenes are believed to be derived from vertebrate proto-oncogenes whose code has been incorporated into infecting retroviral RNA in such a way that it is not normally regulated when reintroduced into inappropriate sites in the DNA of the nucleus, thereby resulting in neoplastic behavior. Some leukemogenic retroviruses do not contain oncogenes. Multiple copies of the provirus are inserted into nuclear DNA, and leukemia develops only in some infected individuals. With this type of virus, development of neoplasia is apparently dependent on chance incorporation of the provirus at an appropriate site.

A retrovirus has been identified which can cause an uncommon form of leukemia in humans. It is designated HTLV-I, for human T cell lymphotrophic virus type I, as it infects T lymphocytes and these cells may evolve into T lymphocytic leukemia-lymphoma. Infection with the virus is endemic in certain parts of the world, such as southern Japan, the Caribbean, South America, and Central Africa. The provirus is randomly incorporated into nuclear DNA, and this does not normally result in neoplastic activity. A second event, yet to be clarified, is responsible for development of malignant properties, and a monoclonal T-leukemic cell population emerges as a result.

GENETIC FACTORS

Occurrence of occasional clusters of cases of leukemia in families suggests genetic factors may be involved, but this is the exception as the disease does not normally occur in an inherited fashion. A number of disorders in which there are inherited abnormalities of nuclear DNA are, on the other hand, associated with an increased incidence of leukemia.

Children with Down syndrome, for example, have an increased incidence of leukemia associated with the presence of the additional chromosome 21 in the cells of the tissues. Inherited disorders such as Fanconi's anemia, Bloom's syndrome, and ataxia telangiectasia are associated with an increased incidence of leukemia, and have in common an increased tendency for chromosome breakage, as occurs in irradiated individuals.

CLASSIFICATION

1. *Chronologic* (based on natural history)
   a. Acute
   b. Subacute
   c. Chronic
2. *Cytologic* (based on predominant cell type)
   a. Undifferentiated blast cell leukemia.
      (hematopoietic reticulum cell: stem cell)
   b. Granulocytic leukemia
      Acute (Myeloblast)
      Chronic (Myelocyte)
   c. Lymphocytic leukemia
      Acute (Lymphoblast)
      Chronic (Lymphocyte)
   d. Monocytic leukemia
      Acute (Monoblast)
      Chronic (Monocyte)
   e. Plasma cell leukemia
   f. 'Lymphosarcoma cell' leukemia.
3. *Functional capacity of release mechanism*
   a. Leukemic
   b. Subleukemic
   c. Aleukemic
4. *FAB (French-American-British) System of Classification of Myeloblastic Leukemia (AML)*

M_1. Myeloblastic leukemia without maturation (AML)
Nongranular blasts with occasional Auer rods or azurophilic granules, 3% or more are myeloper-oxidase positive, no maturational changes.

M_2. Myeloblastic leukemia with maturation (AML)
Maturation to promyelocytic stage: 50% of marrow cells are blasts or promyelocytes; later stages variably present, often with bilobed nuclei, Pelger–Huet anomaly or decreased granulation

M_3. Hypergranular promyelocytic leukemia (APL)
Predominant cell is heavily granulated promyelocytes; bundles of Auer rods common in cytoplasm or free in smear.

M_4. Myelomonocytic leukemia (AMML): Promonocytes plus monocytes comprise 20% of nucleated marrow cells, blood or both; myeloblasts plus promyelocytes are 20% of marrow nucleated cells; monocytic cells have strong nonspecific esterase reaction inhibited by fluoride; esterase activity in myelocytic cells persists after fluoride exposure.

M_5. Monocytic leukemia (AMOL) Granulocytic cells less than 10%, differentiated and poorly differentiated subtypes depend on degree of maturation; esterase reaction inhibited by sodium fluoride.

M_6. Erythroleukemia (EL)
Marrow has 50% erythropoietic forms, often with bizarre morphology or megaloblastic changes (show PAS+ve granules): myeloblasts and promyelocytes 30% or more: abnormal megakaryocytes present.

REVISED FAB CLASSIFICATION (WITH CRITERIA) OF AML

M_0. Minimally differentiated (Fig. 12.2A) Large, agranular blasts (resemble ALL L_2 rarely L1). Myeloperoxidase negative or <3% positive. B-T
lineage markers negative; CD13 and/or CD33 positive; myeloperoxidase positive by immunochemistry or electron microscopy; TdT may be positive.

M_1. Without maturation (Fig. 12.2B)
- Blast cells, agranular and granular types (types I and II) >90% of nonerythroid cells. At least 3% of these are peroxidase or Sudan black positive.
- Remaining 10% (or less) are maturing granulocytes or monocytes.

M_2. With maturation (Fig. 12.2C)
- Sum of agranular and granular blasts (types I and II) from 30–89% of nonerythroid cells
- Monocytic cells <20%
- Granulocytes from promyelocytes to mature polymorphs, >10%.

M_3. Hypergranular promyelocytic (Fig. 12.2D)
- Majority of cells are abnormal promyelocytes with heavy granulation
- Characteristic cells containing bundles of Auer rods ("faggots") invariablly present. Microgranular variant also seen.

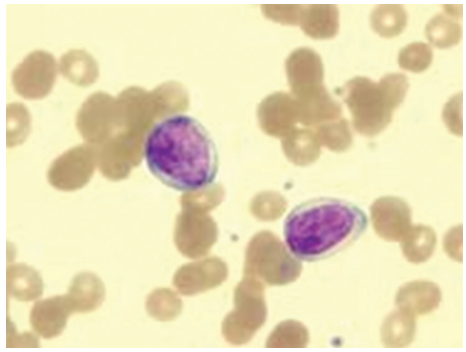


Fig. 12.2A: AML M_0

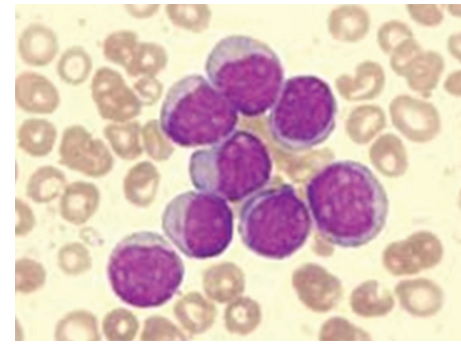


Fig. 12.2B: AML M_1

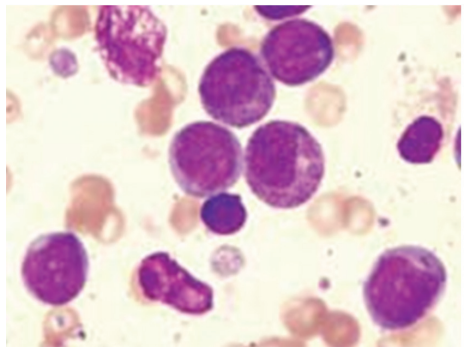


Fig. 12.2C: AML M_2

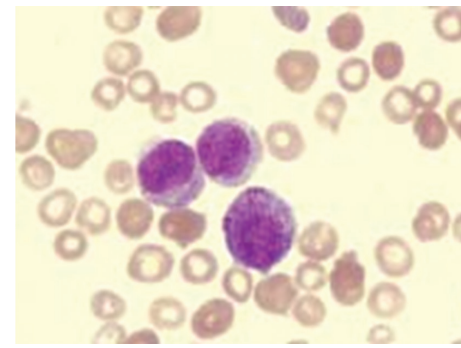


Fig. 12.2D: AML M_3

M_4. Myelomonocytic (Fig. 12.2E)

- In the marrow, blasts >30% of nonerythroid cells
- Sum of myeloblasts, promyelocytes, myelocytes and later granulocytes is between 30 and 80% of nonerythroid cells
- More than twenty percent of nonerytheroid cells are of monocyte lineage
- If monocytic cells exceed 90%, diagnosis is M_5

a. If marrow findings as above and peripheral blood monocytes (all types) >5.0 × 10^9/L, diagnosis is M_4

b. If monocyte count <5.0 × 10^9/L, M_4 can be confirmed on basis of serum lysozyme, combined esterase

c. Diagnosis of M_4 confirmed if >20% of marrow precursors are monocytes (confirmed by special stains)

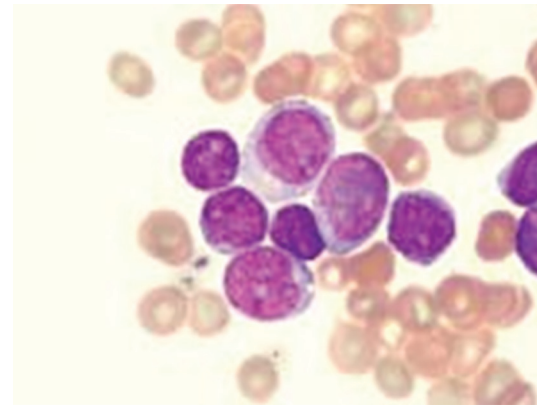


Fig. 12.2E: AML M_4

M_4. With eosinophilia

- Eosinophils >5% of nonerythroid cells in marrow
- Eosinophils are abnormal
- Eosinophils are chloroacetate and PAS positive

M_{5a} Monoblastic (Fig. 12.2F)

- 80% of marrow non-erythroid cells are monoblasts

M_{5b} Monocytic (Fig. 12.2G)

- <80% of monocytic cells are monoblasts, remainder are predominantly promonocytes and monocytes.

M_6. Erythroleukemia (Fig. 12.2H)

- The erythroid component of the marrow exceeds 50% of all nucleated cells
- 30% of remaining nonerythroid cells are agranular or granular blasts (types I an II)
- If >50% erythroid cells but <30% blasts, diagnosis becomes myelodysplastic syndrome

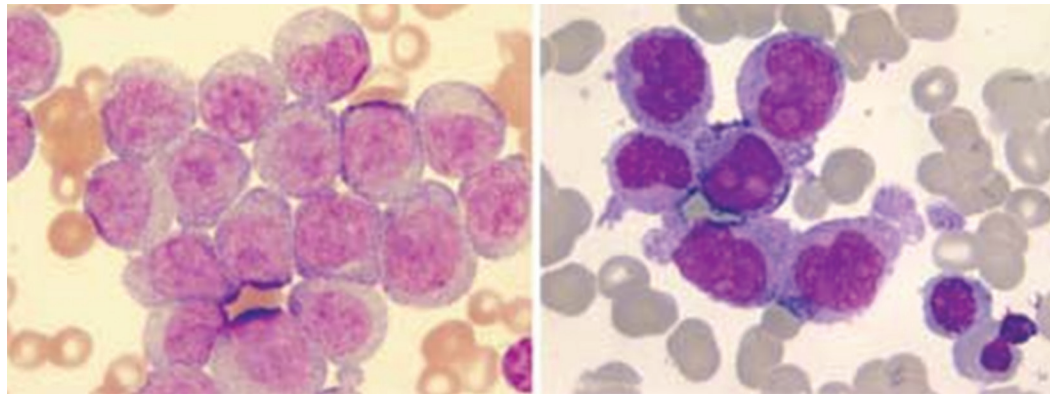


Fig. 12.2F: AML M_{5a}

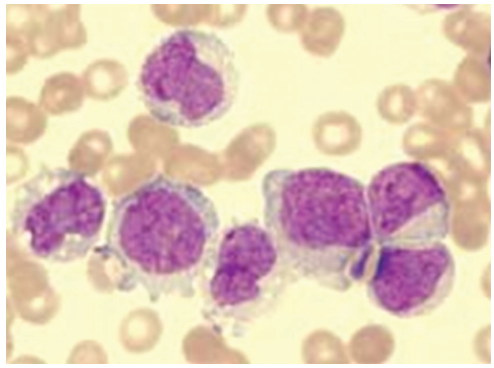


Fig. 12.2G: AML M_{5b}

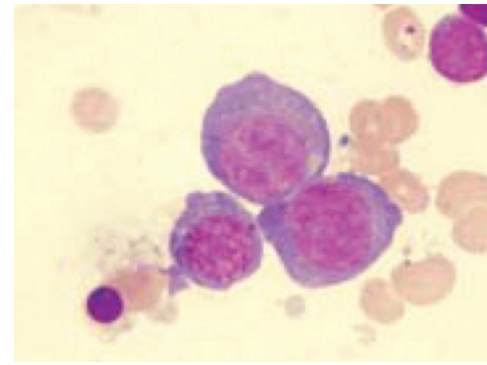


Fig. 12.2H: AML M_6

M_7 Megakaryoblastic (Fig. 12.2I)
- 30% at least of nucleated cells are blasts
- Blasts identified by platelet peroxidase on electron microscopy, or by monoclonal antibodies
- Increased reticulin is common.

- *Variants of Lymphoblastic Leukemia (ALL)* (Table 12.1)

Common (non T, non B)
Thy - ALL (Figs 12.2M and 12.2N)
B-cell ALL

Table 12.1: FAB CIassification of lymphoblastic leukemias

Observation	*L_1 (Fig. 12.2J)*	*L_2 (Fig. 12.2K)*	*L_3 (Fig. 12.2L)*
Consistency of	Homogeneous	Heterogeneous	Homogeneous appearance
Cell size	Uniformly small	Large but variable	Uniformly large
Nuclear shape	Regular, little clefting	Irregular, clefted, indented	Regular rounded
Nucleoli	None or inconspicuous	One or more large	One or more, prominent
Amount of cytoplasm	Scant	Variable, often abundant	Abundant
Other findings	*TdT usuallyincreased	TdT usually increased Myeloperoxidase negative	May have B cell markings

*TdT: Terminal deoxynucleotidyl transferase

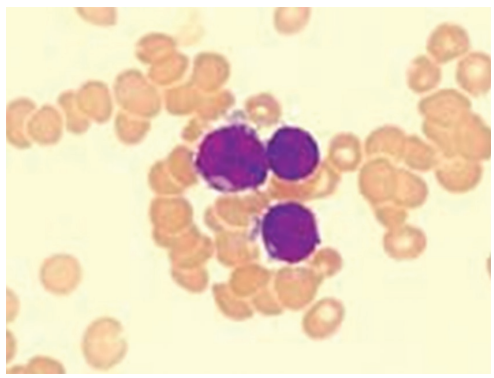


Fig. 12.2I: AML M_7

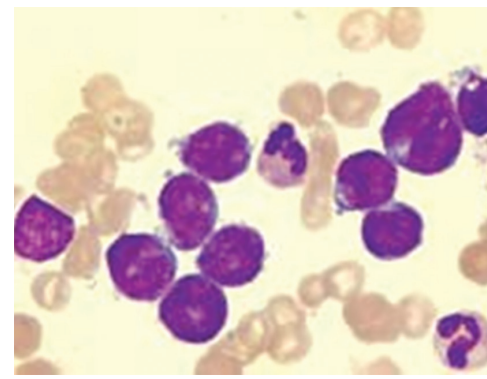


Fig. 12.2J: ALL L_1

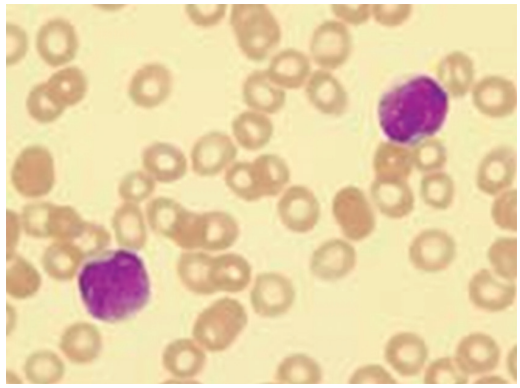


Fig. 12.2K: ALL L_2 (B-cell)

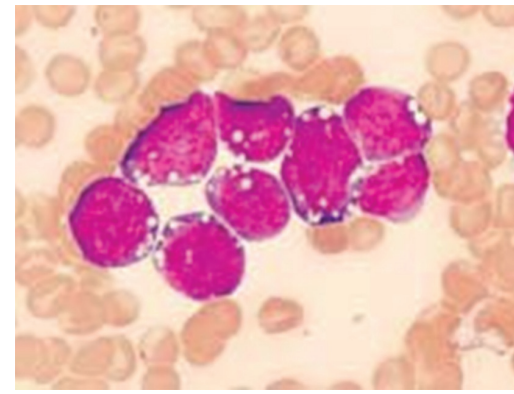


Fig. 12.2L: ALL L_3 (B-cell)

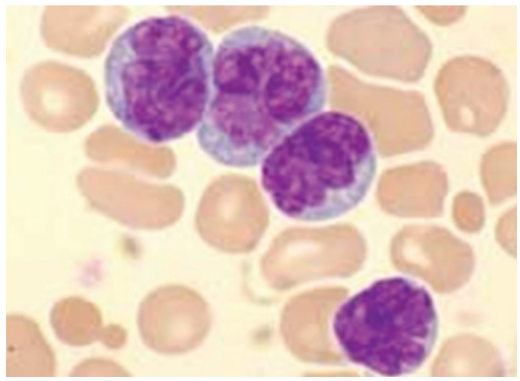


Fig. 12.2M: ALL (T-cell-ALT)

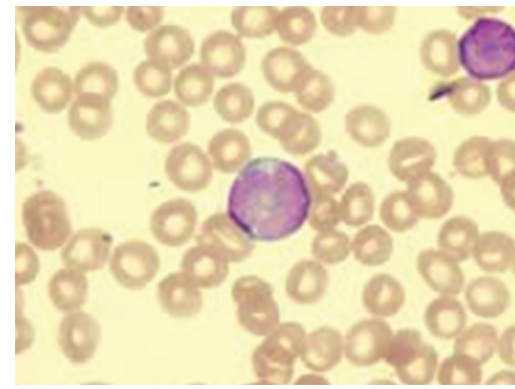


Fig. 12.2N: ALL (T-cell)

Unusual Types

a. Hairy-cell leukemia (HCL) (Fig. 12.2O)
b. Prolymphocytic leukemia (Fig. 12.2P)
c. Dysmyelopoietic syndromes
   - ♦ Chronic myelomonocytic leukemia
   - ♦ Refractory anemia with excess blasts (Fig. 12.2Q).

- *Localized Proliferation of Cells of Same Type*

a. Chloroma (myeloblast)
b. Myeloma (plasma cell)
c. Lymphoma (lymphoblast or lymphocyte).

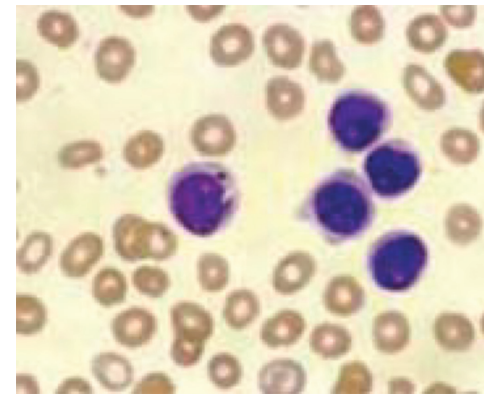


Fig. 12.2O: Hairy-cell leukemia (lymphoblastic)

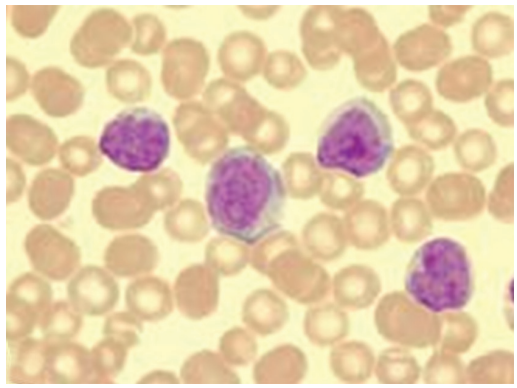


Fig. 12.2P(a): Prolymphocytic leukemia (B-cell)

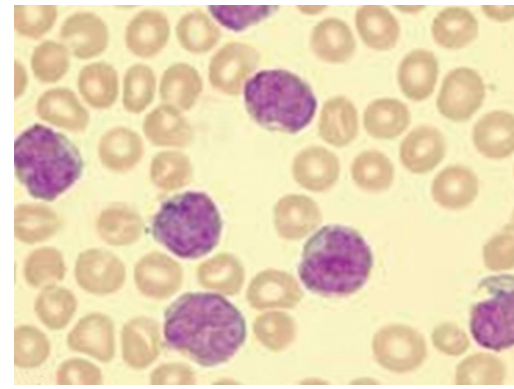


Fig. 12.2P(b): Prolymphocytic leukemia (T-cell)

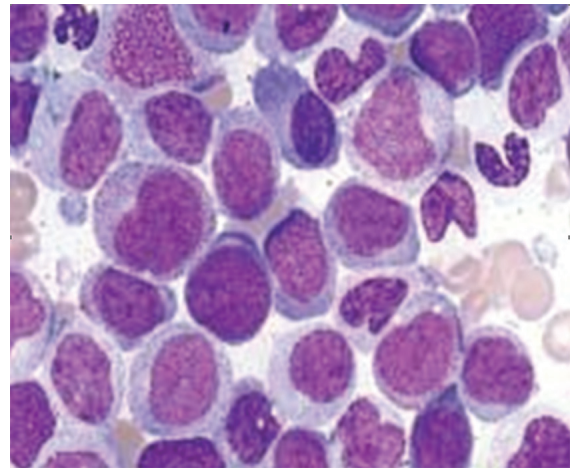


Fig. 12.2Q: Refractory anemia with excess blasts

FAB CLASSIFICATION OF MYELODYSPLASTIC SYNDROMES

1. Refractory anemia (RA) (Fig. 12.2R shows bone marrow picture)
2. RA with ring sideroblasts (Ring sideroblasts >15%) (Fig. 12.2S)

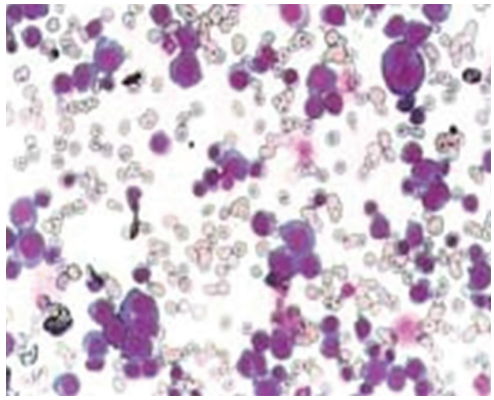


Fig. 12.2R: Refractory anemia, bone marrow

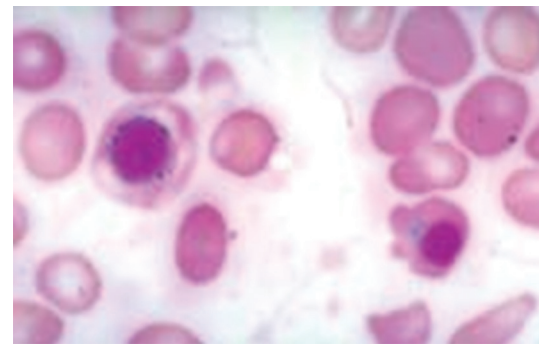


Fig. 12.2S: Refractory anemia with ring sideroblasts

3. RA with excess blasts (RAEB; blasts 5–20%) (Fig. 12.2T)
4. Chronic myelomonocytic leukemia (CMML) (Fig. 12.2U)
5. RAEB in transformation (blasts 20–30%).

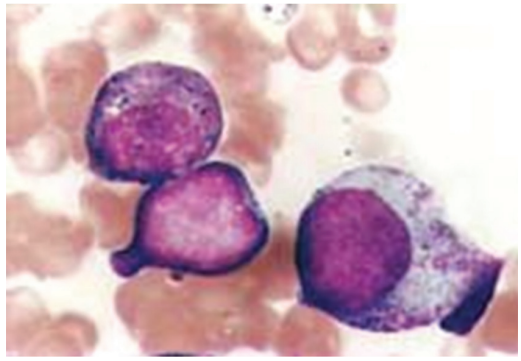


Fig. 12.2T: Refractory anemia with excess blasts (Oil immersion × 1000X)

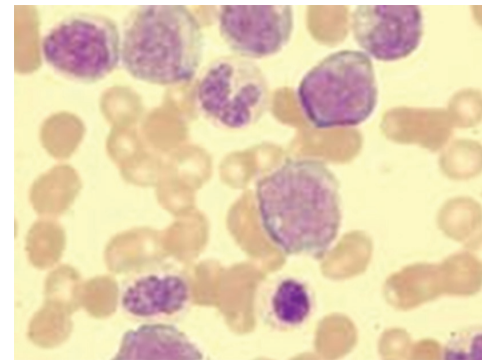


Fig. 12.2U: Chronic myelomonocytic leukemia

ACUTE LEUKEMIAS

COMMONLY FOUND

1. *Acute lymphoblastic leukemia,* (*see* Fig. 12.2J) and
2. *Acute myeloblastic leukemia* (*see* Fig. 12.2B).

The disease may be recognized only when blast cells in the marrow exceed 5% of the cell total. This corresponds to a cell count in excess of 108. When the abnormal cell number approaches 1012 the patient is gravely ill with severe bone marrow failure. Peripheral blood involvement by the leukemic cell and infiltration of organs such as spleen, liver and lymph nodes may not occur until the leukemic cell population comprises 60% or more of the marrow cell total.

The clinical presentation and mortality in acute leukemia arises mainly from—neutropenia, thrombocytopenia and anemia because of bone marrow failure.

INCIDENCE

Acute lymphoblastic leukemia (ALL) is the common form in children. The incidence is highest at 3–5 years, falling off by 10 years. There is a secondary rise after 40 years.

Acute myeloblastic leukemia (AML) occurs in all age groups but more common in adults and less so in children.

CLINICAL FEATURES

Age: Acute leukemia may occur at any age. In children the incidence is highest in the first six years of life; in adults, it occurs at all ages and is not uncommon in middle-aged and elderly persons. Acute leukemia in children, especially young children, is usually lymphoblastic in type, while in adults it is usually myeloid.

Clinical picture: The clinical picture in the principal types is largely indistinguishable, although certain features such as gum hypertrophy and ulcerative lesions of the rectum and vagina are more common in the myelomonocytic (M_4) than in the other types. Lymph node enlargement is more common in lymphoblastic leukemia.

Symptoms: The onset of symptoms may be abrupt or insidious. In general, an abrupt onset is more common in children and young adults. The most common mode of presentation is with symptoms of anemia or hemorrhage, infective lesions of the mouth and pharynx, fever, prostration, headache, and malaise. Presenting problems may occur either singly or in combination, and are listed below. In about one-half of all cases of acute leukemia in childhood there is a history of infection, frequently respiratory, antedating the apparent onset of leukemia by several weeks or months.

Hemorrhagic tendencies such as skin petechiae and bruises are common. Bleeding from the gums and nose is also common and persistent bleeding after tooth extraction or tonsillectomy occasionally first brings the condition to notice. Gastrointestinal, renal tract, uterine, and nervous system hemorhage commonly occur in the course of the disease. Impairment of vision and deafness or vertigo may result from hemorrhage into the eye and ear respectively. There is a relation between bleeding and fever, the onset of fever sometimes being accompanied by the first appearance of hemorrhage, or by an increase in its severity. Sudden onset of a profound hemorrhagic tendency may be due to development of disseminated intravascular coagulation, a condition associated with acute promyelocytic leukemia (M_3) (Table 12.2).

Table 12.2: Clinical presenting manifestations of acute leukemia

Common
• Anemia
• Fever, malaise
• Hemorrhage, bruising, petechiae
Less common
• Infection of the mouth and pharynx
• Pains in bones and joints (childhood especially)
• Upper respiratory tract infection (childhood especially)
• Superficial lymph node enlargement (childhood especially)
Occasional
• Abdominal pain
• Mediastinal obstruction (childhood especially)
• Nervous system abnormalities
• Skin rash
• Gum hypertrophy

Oral and pharyngeal infective lesions are frequent. The patient may complain of a sore throat, ulceration of the gums, mouth, or pharynx, or an upper respiratory tract infection. Patients with marked oral sepsis or gingival hypertrophy may first consult a dentist. Lesions in the mouth and pharynx vary in severity from small necrotic ulcers to areas of marked swelling, with extensive necrosis and ulceration. These lesions can be extremely painful. The gums are frequently infected, and necrosis, ulceration, and bleeding may be present. In a few cases there is true hypertrophy of the gingivae, with marked swelling and heaping up of the gum margin so that the teeth appear almost buried in the gums. This type of gingival hypertrophy is especially characteristic of myelomonocytic (M_4) and monocytic leukemia (M_5), although it does occur less commonly in the other forms. It represents tissue infiltration by leukemia.

Infections are common and may be the presenting feature. They include upper and lower respiratory tract infection, cellulitis, paronychia, bacteremia, and otitis media. Respiratory tract infection is particularly prominent in children. Susceptibility to infection is due mainly to neutropenia, but a diminished immune response plays a contributing role.

Constitutional symptoms such as fever, malaise, rigors, prostration, and generalized aches and pains are common, especially in patients with infection. However, they may occur in the absence of an obvious site of infection, in which case blood cultures should be performed, as septicaemia may be present. Fever can be especially high in children, temperature 39–41°C being not uncommon.

The liver and spleen can be slightly to moderately enlarged. Enlargement of the spleen below the umbilicus can occur, but is unusual. Enlargement tends to be more pronounced in children than in adults. Ulceration, or even infiltration of the alimentary tract, occasionally results in abdominal pain or diarrhea. Ulcerative lesions of the rectum and vagina, anal fissures, and perianal abscesses are relatively common.

The lymph nodes may be slightly or moderately enlarged, especially in lymphoblastic leukemia. However, in many cases they are not palpable, or are only very slightly enlarged when the patient first presents. Enlargement tends to be more pronounced in children. Because of the frequency of oral and pharyngeal sepsis, the cervical nodes are more often enlarged than nodes in other areas, and they are frequently tender.

Pain and tenderness in the bone and about the joints may occur, especially in children. Tenderness of the sternum, most marked over the lower end, is occasionally present. Apart from tenderness, there is usually no clinical evidence of bone involvement. In a few cases, the clinical picture of acute osteomyelitis is simulated by the presence of swelling, redness, and tenderness of bones, usually close to the joint. Joint manifestations include migratory joint pain, persistent pain in one or more joints, and the local manifestations of heat, redness and swelling. The bone pain in children may cause them to stop walking. This picture may resemble acute rheumatic fever. X-ray changes in bones may be present, particularly in children, and consist of destruction of the cortex with thinning and erosion, and periostitis, with periosteal elevation and the formation of new subperiosteal bone, most frequently at the metaphyseal ends of the long bones. Epiphyseal growth may be disturbed.

The most common finding is the *cardiovascular system* is tachycardia, a result of either anemia or infection, or a combination of both. Pericarditis may occur due to hemorrhage, infiltration with leukemic cells, or viral or bacterial infection.

Involvement of the central nervous system results from either hemorrhage, infection, or infiltration with leukemia, the latter being a common problem in children. Intracerebral hemorrhage represents a very serious event, occurring especially in patients with a rapidly rising white cell count, profound thrombocytopenia, or disseminated intravascular coagulation.

Meningeal involvement (meningeal leukemia) was seen very commonly in children, often during hematological recession. More adults with acute leukemia are achieving long remissions, and as a result an increasing incidence is being observed in this group. Meningeal leukemia is manifested by signs of raised intracranial pressure, with headache, vomiting, papilloedema, meningismus, and irritability; these can occur singly or together. Cranial nerve palsies may develop. The cerebrospinal fluid often is at increased pressure, with an elevated content of proteins and cells. Careful examination of the cells with appropriate staining on cytocentrifuge preparations frequently provides the diagnosis. The possibility of meningeal leukemia should be considered in a patient, especially a child, who develops unexplained headache or vomiting, either in remission or relapse. Less frequently, the patient presents with focal neurological signs. Occasionally, signs of spinal cord compression develop, which may progress to paraplegia.

Due to Bone Marrow Failure

1. Pallor, lethargy, dyspnea from anemia.
2. Fever, malaise, features of mouth, throat, skin, respiratory, perianal or other infections including septicemia are common usually due to:
   - Gram-negative gut bacteria
   - Staphylococci and streptococci
   - Viral—Herpes simplex and zoster
   - Fungal—*Candida*
   - Protozoal—*Pneumocystitis carinii.*
3. Spontaneous bruises, purpura, bleeding gums and bleeding from venepuncture sites because of thrombocytopenia are common. Occasionally, there may be major internal hemorrhage.

Due to Organ Infiltration

1. Tender bones especially in children.
2. Superficial lymphadenopathy in ALL.
3. Moderate splenomegaly, hepatomegaly especially in ALL.
4. Gum hypertrophy and infiltration, rectal ulceration, skin involvement (particularly in myelomonocytic and monocytic types).
5. Meningeal syndrome (particularly in ALL)—headache, nausea vomiting, blurring of vision and diplopia. Fundal examination may reveal papilledema and sometimes hemorrhage.
6. Other occasional manifestations of organ infiltration include testicular swelling in ALL or signs of mediastinal compression (also in ALL).

LABORATORY FINDINGS

Routine Hematologic Investigations

1. A normochromic normocytic anemia.
2. The white cell count may be decreased, normal or increased upto at least 500×10^9/L.
3. Thrombocytopenia in most cases, often extremely low in AML.
4. *Blood film examination* shows variable numbers of blast cells, in AML the blasts may show Auer rods and other abnormal cells may be present, e.g. promyelocytes, myelocytes, agranular neutrophils, pseudopelger cells, myelomonocytic cells in erythroleukemia many normoblasts may be seen but these may be seen in smaller numbers in the other forms.
5. The *bone marrow* is hypercellular with a marked proliferation of leukemic blast cells which typically amount to over 75% of the marrow cell total.

Differentiation of ALL and AML

In most cases the clinical features and morphology on routine staining separates ALL from AML. In ALL blasts show no differentiation whereas in AML some evidence of differentiation to granulocytes is often seen in the blasts or their progeny. Special tests are needed when cells are undifferentiated (Tables 12.3 and 12.4).

Table 12.3: Morphologic clues for differentiation of blasts in AML and ALL

	AML Myeloblast	*ALL Lymphoblast*
Blast size	Large blast	Small blast
Cytoplasm	Moderate	Scant
Chromatin	Fine, lacy	Dense
Nucleoli	Prominent (usually >2)	Indistinct (usually <2)
Auer rods	Present in 10–40% cases	Never present

Table 12.4: Cytochemistry		
	ALL	*AML*
Cytochemistry Myeloperoxidase	–	+ (including Auer rods)
Sudan black	–	+
Nonspecific esterase	–	+ in monocytic types
Periodic acid–Schiff (PAS)	+ (coarse)	+ (fine)
Acid phosphatase	+ (T cell ALL)	–

Special Investigations

Tests for disseminated intravascular coagulation (DIC) are positive in promyelocytic leukemia. Lumbar puncture shows raised spinal fluid pressure; it contains leukemic cells in patients with meningeal leukemia.

Differential Diagnosis

One must differentiate from other disorders which have a similar clinical picture, and form disorders with a blood picture resembling acute leukemia.

DISORDERS WITH SIMILAR CLINICAL FEATURES

Other Reasons for Pharyngeal Ulceration

An important problem is differentiation from the infectious mononucleosis, as in both conditions fever, ulceration of the throat, lymphadenopathy and splenomegaly are associated with the appearance of atypical white cells in the blood. Other causes of ulceration of the throat are acute tonsillitis, Vincent's angina, diphtheria and agranulocytic angina, which are differentiated by hematological and microbiological investigation.

Other Reasons of Arthralgia and Bone Pain

A possible diagnosis of rheumatic fever may be considered in children presenting with joint pains, as the two conditions have a number of features in common, including joint pains, fever, pallor, anemia, a systolic bruit, epistaxis, and tachycardia. Polymorph leukocytosis is usual in acute rheumatic fever, whereas neutropenia is the rule is acute leukemia. Occasionally, when there is marked reddening, swelling, or tenderness of one jont, osteomyelitis may be suspected, especially in children.

Other Reasons of Fever and Malaise

These include subacute bacterial edocarditis, influenza, upper respiratory tract infections, septicemia, typhoid fever, brucellosis, and lymphoma.

Cases presenting with acute abdominal pain, mediastinal obstruction, skin rash, or nervous system involvement must be differentiated from other causes of such conditions.

DISORDERS WITH A SIMILAR BLOOD PICTURE

Myelodysplastic syndromes: Significant degrees of anemia, neutropenia and thrombocytopenia, accompanied by blast cells in the peripheral blood, commonly occur in these disorders, which may be effectively distinguished from acute leukemia only by a lower proportion of blast cells in the bone marrow aspirate.

A leukemoid blood picture. The blood picture in infectious mononucleosis is the one most commonly confused with that of acute lymphoblastic leukemia. Other leukemoid

blood pictures simulating acute leukemia are uncommon, occurring rarely in infections, including tuberculosis, where the pattern of myeloid precursors in the blood is more in keeping with less fulminant forms of leukemia.

Other causes of pancytopenia must be differentiated from 'subleukemic' acute leukemia in which few or no blast cells are observed in the peripheral smear.

COURSE AND PROGNOSIS

Prior to the introduction of antileukemic therapeutic agents, survival varied from a few weeks to about 10 months, with an average of about 20 weeks in unequivocal acute leukemia. Longer survivals of 12 months or more occur in untreated disorders which formerly have been classified as acute leukemia, but would now be designated as myelodysplastic conditions using FAB classification criteria. Spontaneous remissions are very rare, and current experience would indicate the proportion is very much less than considered to be the case four decades ago.

In untreated disease, anemia is an outstanding feature but can usually be corrected without difficulty by red cell transfusions. It is the infective and hemorrhagic manifestations that eventually produce the major clinical problems, and ultimately are directly responsible for death in the majority of patients.

An important factor influencing prognosis is whether sustained complete remission is attained by treatment. Prognosis has been greatly altered in childhood lymphoblastic leukemia by the introduction of effective measures for eradicating disease from the bone marrow and central nervous disease from the bone marrow and central nervous system. Average survival is greatly extended, and in the L1 form the majority are free of disease five years after presentation. Outcome is less satisfactory in other subtypes of acute leukemia, including all forms encountered in adults, but the frequency of response to chemotherapeutic agents is sufficiently high to raise the prospect of more prolonged remissions with the introduction of more effective management programs.

TREATMENT

Treatment is considered in the following order:

- General considerations
- Specific therapeutic agents
- Therapy in the individual patient
- Symptomatic and supportive therapy.

General Considerations

Conservative approaches to the management of acute leukemia have now given way to aggressive combination chemotherapy, combined with antibiotic supportive measures and platelet transfusions to combat infection and bleeding, respectively. Such remission-induction treatment entails a high degree of morbidity at the time, but the significant prolongation of life that follows successful remission induction, coupled with the greatly improved quality of life during complete remission, is the reason why it has become accepted as the standard approach to management in the absence of overriding considerations, such as extremes of age or infirmity due to other conditions which have a poor prognosis in their own right. Attainment of complete remission entails loss of all abnormal clinical and routine diagnostic laboratory findings attributable to the leukemia, and return to good health. Peripheral blood and bone marrow parameters return to normal, and therefore morphologically normal blast cells make up lesser than 5% of cells in the

bone marrow aspirate. Substantial but less extensive improvement is classified as partial remission when there is return to reasonable physical health, with significant improvement in abnormal physical signs attributable to the leukemia, a return of at least two of the three peripheral blood elements to normal values, and a significant reduction in the degree of leukemic cell infiltration in the bone marrow.

The *objects of treatment* are eradication of the leukemic process and control of complications, especially those due to inadequate production of normal blood cells, such as anemia, infection, and hemorrhage.

Eradication of the leukemic process involves the use of antileukemic agents, usually in combination, with supplementary measures such as irradiation for destroying disease in sanctuary sites such as the central nervous system. In many forms of acute leukemia, the amount of neoplastic tissue is greatly reduced, but not completely eliminated by treatment, and relapse occurs in many instances after a phase of hematological normality described as complete remission. Considerable efforts are made to prevent relapse of disease, and this aspect of management is currently the subject of intensive investigation.

Treatment centers: Treatment should be carried out by highly experienced personnel in hospitals with appropriate supportive facilities. This permits the most effective use of anti-leukemic agents in a setting where complications of the drugs, especially severe marrow depression. Optimum management is unfortunately beyond the capacity of the conventional general medical hospital unit.

Response to treatment is variable and it varies with the category of leukemia, and from case to case, so that is not possible to predict with precision the nature or duration of a response in the individual patient. Factors that influence outcome in adults treated by current chemotherapy programmes are given in Table 12.5 and do not take into consideration the impact that bone marrow transplantation is beginning to make in selected categories of this disease.

Table 12.5: Treatment response in adult acute leukemia

	Acute myeloid leukemia	Acute lymphoblastic leukemia
Rate of remission	60–80%	70–90%
Median duration of remission	Under 2 years	2 years
Prolonged disease-free remissions (>3 years disease-free)	10–40%	20–40%
Lower likelihood of remission	Increasing age Prior myelodysplastic or myeloproliferative disorder Certain types of chromosomal abnormality Prior irradiation or alkylating agent treatment	Increasing age Presence of chromosomal abnormality, certain types in particular High TLC Mediastinal mass
Lower likelihood of prolonged remission	Increasing age High initial TLC, FAB M5, M6 subtype Slow clearance of blasts High initial LDH and fibrinogen Certain types of chromosomal abnormality	Increasing age High initial TLC B and T surface markers present FAB L3 subtype

It is evident that the age of the patient and the morphological type of leukemia are important in influencing the probability of obtaining complete remission. These two factors are to some extent related, as acute leukemia in children is much more frequently of the L1 lymphoblastic variety than in adults, and in this form the best responses to treatment are obtained. Children under the age of two years do not respond as well as older children. Complete remissions are obtained in more than 90% of children with acute lymphoblastic leukemia. Remission is less readily achieved in the less common childhood acute myeloid leukemia. In adults, complete remissions are obtained in about 70% of cases overall. Remissions occur more readily in the lymphoblastic than in the myeloid forms, but there is now no subclass of acute leukemia in which useful remission rates have not been reported. Those in which remission is least readily achieved or sustained include acute leukemia evolving during the course of myeloproliferative disorders, myelodysplastic disorders, and chronic myeloid leukemia, and secondary to irradiation or alkylating agent therapy.

Thus the important consideration in assessment of outcome of any particular form of therapy is the nature of the leukemic process, as this can radically affect response to current treatment programs. An improved prognosis in a particular subtype can also be related to introduction of more effective management strategies. For example, after attainment of complete hematological remission in L1 acute lymphoblastic leukemia in children, addition of steps to treat disease sequestered in the central nervous system by radiotherapy and/or chemotherapy has improved life expectancy. Figures are now being reported of greater than 50% 5-year leukemia-free survival in this particular type of acute leukemia, and it appears likely that many children free from disease after 5 years will not undergo subsequent relapse.

Outcome of treatment is currently less encouraging in adults. There has, however, been steady improvement since the introduction of treatment with cytosine arabinoside plus daunorubicin, with an increase in median survival to between 18 months and 2 years, and a significant subpopulation of long-term survivors has been accruing since as early as 1968. There are also indications that allogenic and autologous bone marrow transplantation performed after induction of complete hematological remission can, in certain categories of acute leukemia, prolong the duration of remission, and thus prolong survival, as relapse of the disease is usually associated with a less satisfactory response to the initially successful treatment regimen.

Specific Therapeutic Agents

Usually employed agents currently in routine use for induction of remission and for the attempted prolongation of remission are summarized in Table 12.6. Remission-induction therapy is most commonly carried out by administration of a combination of drugs, as this has been found in practice to result in synergism of toxic action on leukemic cells, as indicated by increased incidence of remission, coupled with reduced toxic side-effects when drugs with differing modes of action are employed. There are also theoretical advantages in the early introduction of several different agents, either at the same time or in close sequence, for obtaining the maximum degree of leukemic cell killing by elimination of subpopulations of cells that are insensitive to one agent but not another. Less commonly, in clinically indolent disease or in subjects considered unlikely to tolerate intensive combination chemotherapy, treatment with a single agent is administered.

The most effective drugs in the treatment of acute myeloid leukemia are cytosine arabinoside and the anthracycline antibiotics, daunorubicin and doxorubicin (adriamycin).

A relatively recent addition, amsacrine (m-AMSA), is also effective and acts with similar efficacy to the anthracyclines in combination with cytosine arabinoside (with or without 6-thioguanine) (Table 12.5).

Cytosine arabinoside is effective in certain forms of leukemia when administered intravenously in low dosage regimens of approximately 10–20 mg/m^2/day over extended periods of up to 3 weeks. The conventional dosage is of the order of 100 mg/m^2/day over 5–7 days, and improved results are considered to be achieved when delivery is by continuous intravenous infusion, although very similar results are obtained by twice-daily subcutaneous injections in ambulatory outpatients. Bone marrow depression is related in degree to dosage and duration of therapy. High dosage treatment of the order of 3000 mg twice daily for 3–6 days has a potent effect on leukemia, and may produce remission in disease which is unresponsive to conventional doses of cytosine arabinoside. Such high dosage treatment is accompanied by a high incidence of cerebellar toxicity, conjunctivitis, fever, rash, mucositis, arthralgia, hepatic dysfunction, as well as prolonged severe *bone marrow depression*.

Daunorubicin is a highly effective anthracycline which is administered intravenously, with considerable caution, as extravasation is associated with local tissue necrosis. It also produces marrow depression and mucositis. A particular problem associated with this drug and the related anthracycline, adriamycin (doxorubicin), is cumulative cardiotoxicity, which can cause irreversible cardiomyopathy when the total dose exceeds about 500 mg/m^2. Cardiotoxic effects are more problematic in subjects with subnormal myocardial functional reserve, and for this reason these drugs are avoided in the elderly and in subjects with cardiac disease. Elimination of the drug is decreased in hepatic insufficiency, when conventional doses can also cause more serious side effects.

Thioguanine and mercaptopurine are orally administered agents, degraded by xanthine oxidase, and thus less effectively eliminated when given concurrently with allopurinol, a situation associated with potentially serious toxic effects unless the dosage is appropriately reduced.

Amsacrine (m-AMSA) is approximately as effective in acute myeloid leukemia as the anthracyclines when administered in combination with cytosine arabinoside. The drug is a synthetic acridine analogue which intercalates with DNA, preventing

DNA from serving as a template for DNA replication or transcription. Severe myelosuppression and mucositis are major side-effects, but an advantage over anthracycline drugs is that m-AMSA is not characteristically associated with cumulative cardiotoxicity.

Acute lymphoblastic leukemia displays a somewhat different pattern of responsiveness, and vincristine corticosteroids, anthracyclines, and L-asparanginase are the most useful drugs currently available. Other agents used to treat this type of leukemia include cytosine arabinoside and methotrexate (Table 12.6).

Table 12.6: Treatment of acute leukemia—drugs in established use

Drug	*Probable pharmacological action*	*Usual mode of administration*	*More common toxic effects*
Cytosine arabinoside (Cytarabine)	After phosphorylation, acts as competitive antagonist of pyrimidine nucleotide synthesis and DNA polymerase	Intravenous or subcutaneous infusion or injection	Nausea, marrow depression, rash, arthralgia

Contd.

Table 12.6: Treatment of acute leukemia—drugs in established use

Drug	*Probable pharmacological action*	*Usual mode of administration*	*More common toxic effects*
Daunorubicin	Antibiotic produced by *Streptomyces coeruleorubidus* (*Streptomyces peuceticus*) Inhibits replication and transcription of DNA	Intravenous injection	Marrow depression, cardiotoxicity, alopecia, nausea, vomiting
Doxorubicin (Adriamycin)	C-14 hydroxylated daunorubicin derivative	Intravenous injection	As for daunorubicin
6-Thioguanine and 6-Mercaptopurine	Purine analogues which interfere with *de novo* nucleotide metabolism, salvage, and reutilization pathways	Oral	Nausea, vomiting, marrow depression
Amsacrine (m-AMSA)	Synthetic amino acridine, intercalates with DNA, preventing DNA replication and transcription	Intravenous injection	Nausea, vomiting, mucositis, marrow depression, alopecia
Vincristine	Alkaloid of complex structure derived from Vinca rosea. Acts probably by disruption of the mitotic spindle	Intravenous injection	Motor and sensory peripheral neuropathy, alopecia, abdominal pain and constipation
Prednisolone or prednisone	Synthetic derivative of hydrocortisone. Cause of cytolysis in neoplastic lymphocytic cells unclear	Oral	Susceptibility to infection, mineralo-corticoid effects, diabetes, activation of peptic ulcer, osteoporosis
L-Asparaginase	Enzyme derived from micro-organisms, degrades L-asparagine to aspartate and ammonia. Asparagine essential to some malignant cells	Intravenous injection	Anaphylaxis and other hypersensitivity reactions, pancreatitis, hepatic dysfunction
Methotrexate	Folic acid analogue. 4-amino-N_{10}-methyl-pteroylglutamic acid. Blocks folate reductase and inhibits DNA synthesis by preventing production of folinic acid	Intravenous infusion or oral	Mucositis, diarrhea, marrow depression, occasionally alopecia, hepatic fibrosis

Therapy in the Individual Patient

The basic aims of antileukemic therapy are to induce a remission and to prolong the duration of remission.

Generally, different agents are used in the attempt to induce remission in childhood and in adult acute leukemia. This is related largely to the difference, as previously discussed, in the types of leukemia encountered in these two age groups, with the L1 form of acute lymphoblastic leukemia predominating in children and constituting only a very small component of cases of acute leukemia in adults. There is a tendency for children to be treated as for acute lymphoblastic leukemia, and for adults to be treated as for myeloid leukemia, when the morphological type of the leukemia is unclear.

CHILDHOOD ACUTE LEUKEMIA

Acute Lymphoblastic Leukemia

Induction of remission: Administration of vincristine and prednisolone has been a widely accepted means of remission induction, and results in remissions in about 90% of cases. It has become increasingly apparent that for remissions to be sustained in a greater proportion of patients, additional leukemic cell kill during induction therapy is required. To this end an anthracycline (daunorubicin or doxorubicin), or L-asparaginase, or both, is added to the vincristine and prednisolone, as in the representative treatment program shown in Fig. 12.3.

A dramatic fall in the number of circulating blast cells may occur within 2–3 days in responsive cases, accompanied by reduction in the degree of splenomegaly and lymphadenopathy. Lysis of the malignant cells is sometimes sufficiently rapid to result in profoundly increased production of uric acid. This can cause rapid onset of impaired renal function, with a rapid rise in plasma creatinine, potassium, and phosphate levels, sometimes accompanied by a correspondingly profound depression of the calcium levels. Such rapid and extreme electrolyte disturbances are conducive to promotion of cardiac arrhythmias, which are potentially fatal.

Prophylactic measures should always be instituted before and during remission induction therapy for this reason, a consideration of especial importance when the blood count of leukemic cells is high, or marked splenomegaly or lymphadenopathy is present. These measures include administration of allopurinol in order to depress urate production. Administration of allopurinol is dangerous in subjects receiving 6-mercaptopurine, unless due allowance is made in the dosage of 6-mercaptopurine for the inhibitory action of allopurinol on its catabolism. Another important step under circumstances where there can be rapid cell lysis in response to therapy is the institution of increased fluid input to facilitate removal of purine degradation products in the urine. The solubility of urate can be increased by alkalinization of the urine, so that the likelihood of insoluble urate

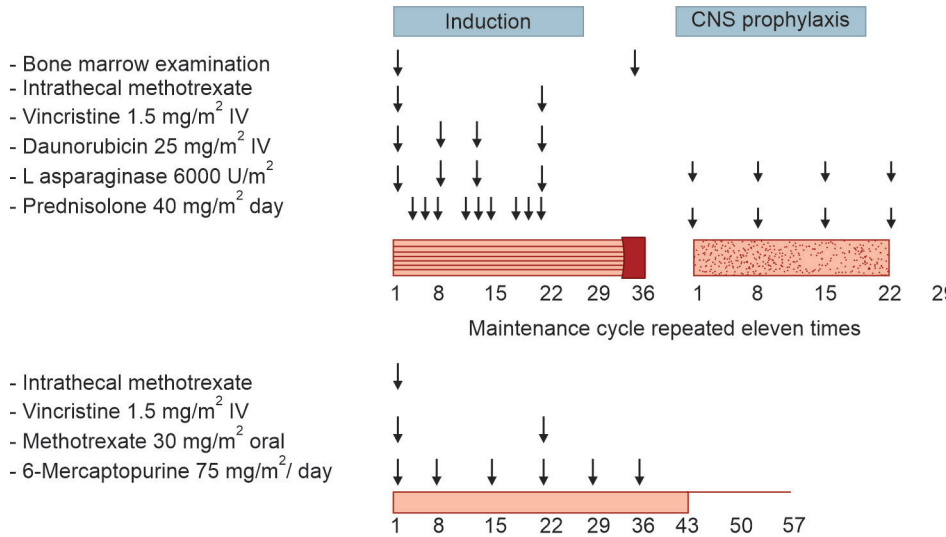

Induction
CNS prophylaxis
- Bone marrow examination
- Intrathecal methotrexate
- Vincristine 1.5 mg/m2 IV
- Daunorubicin 25 mg/m2 IV
- L asparaginase 6000 U/m2
- Prednisolone 40 mg/m2 day
1 8 15 22 29 36 1 8 15 22 29
Maintenance cycle repeated eleven times
- Intrathecal methotrexate
- Vincristine 1.5 mg/m2 IV
- Methotrexate 30 mg/m2 oral
- 6-Mercaptopurine 75 mg/m2/ day
1 8 15 22 29 36 43 50 57

Fig. 12.3: An example of a protocol used for treatment of acute lymphoblastic leukemia in children. Note the combination of different types of active agent, and the routine prophylactic treatment of the central nervous system

deposition in the renal tubules under these circumstances is reduced. Prophylatic measures of this kind should be instituted during treatment of any neoplastic process where rapid lysis of neoplastic cells is a possibility.

The degree to which the counts of neutrophils and platelets is depressed specifically by myelosuppressive effects of treatment is to a considerable extent related to the nature of the agents employed for remission induction, and to the duration that they are administered there may be relatively little further depression over that already produced by the disease process when prednisolone and vincristine are employed. Myelosuppression is generally much more severe with daunorubicin, doxorubicin, and cytosine arabinoside.

Effects of chemotherapy are monitored by changes in the counts of leukemic cells, neutrophils, and platelets in the blood, and by changes in the bone marrow disappearance of leukemic cells from the blood and bone marrow associated with significant hypocellularity of the bone marrow is generally regarded as an indication for a delay in therapy. Persistence of leukemic cells is regarded as an indication for continuation of active treatment. The majority of remissions occur after one or two courses of treatment, and are followed by a progressive rise in the counts of neutrophils and platelets, and the level of hemoglobin, to normal values.

Maintenance Therapy

Such treatment is usually commenced after the blood picture has returned essentially to normal after induction of complete remission. The driving ambition of the therapist is selectively to eliminate the leukemic process. A particularly important step in proceeding towards this objective has been the introduction of treatment, after hematological remission has been achieved, to destroy residual leukemic cells sequestered in the central nervous system. CNS leukemia is particularly common in childhood acute lymphoblastic leukemia, and the striking improvement in survival that has followed the introduction of prophylactic treatment to prevent this complication argues strongly for its use as the next step in management following remission induction. Treatment is usually commenced within one month of attaining remission, often by irradiation of the cranium with 24 Gy over 3–4 weeks. Lower doses are used in children under the age of two years. Such treatment, of course, fails to irradiate leukemic cells in the spinal cord, and for this reason intrathecal methotrexate is given for five doses, twice weekly during the course of cranial irradiation. Approximately the same results are achieved by intrathecal chemotherapy given with sufficiently high doses of parenteral chemotherapy to penetrate the CNS, in an attempt to avoid undesirable effects of cranial irradiation.

The other basic concept underlying the approach to therapy after induction of remission is administration of further systemic chemotherapy in an attempt to eradicate or continually suppress any residual leukemic cells. One approach has been to administer large doses of chemotherapeutic agents as so-called 'consolidation' therapy. This may have merit, especially when the type of acute leukemia is associated with poor prognostic factors, such as L3 morphologic features, T cell surface antigens, the presence of the Philadelphia chromosome, etc. and the regimen often includes drugs not included in the initial induction therapy.

Present evidence indicates that very expensive chemotherapy is of lesser importance in children who have disease with good prognosis and have been managed with prophylactic CNS treatment, where good results are achieved by maintenance therapy consisting of daily 6-mercatopurine given by mouth in a dose of 50 mg/m^2, supplemented by a weekly dose of methotrexate, 20 mg/m^2, also given by mouth. On such a regimen, the drug dosage is

modified to avoid depression of the leukocyte count below 3×10^9/L. Intermittent courses of vincristine and prednisolone may also be added. Alternative regimens for maintenance chemotherapy have been proposed.

Once children who have received prophylactic CNS therapy for leukemia reach three years without evidence of relapse, there is little evidence that further cytotoxic therapy provides any advantage. Relapse can, however, occur during the phase of maintenance treatment, and management depends on the previous therapeutic history of the patient. If prophylactic CNS therapy was not given, and remission was readily obtained in the first instance with prednisolone and vincristine, maintenance therapy is withheld and the patient treated as on the first occasion. However, if difficulty was experienced in achieving a remission on the first occasion, or remission is not readily obtained with retreatment, additional agents such as daunorubicin, cyclophosphamide or L-asparaginase should be introduced. A second remission is usually obtained, but remission duration tends to be shorter than following the first remission, and progressively increasing difficulty is experienced in achieving subsequent remissions. Maintenance therapy during second and subsequent remissions should include, where possible, additional chemotherapeutic agents, and if CNS prophylaxis had not been previously administered, this should be performed as leukemic cells harboured within the CNS may have been the origin of the relapse. Another option in the treatment of children whose disease has relapsed and undergone successful reinduction is allogeneic bone marrow transplantation, if a suitable donor is available. The relapse rate following allogeneic bone marrow transplantation under these circumstances is comparatively high (25–50%), but less than the much higher rate in children treated with chemotherapy alone.

Acute Myeloid Leukemia

The basic principles of treatment are the same as for acute myeloid leukemia in adults and are discussed in the following section.

ADULT ACUTE LEUKEMIA

Roughly 20% of cases in adults consist of acute lymphoblastic leukemia, and in subjects over the age of 25 years, the disease does not respond to treatment as well as acute lymphoblastic leukemia in children. The remaining cases consist of various categories of acute nonlymphoblastic leukemia, and for convenience in this section are referred to collectively as acute myeloid leukemia, although it is recognized that the various categories possess specific properties, including different patterns of response to chemotherapy.

Acute Lymphoblastic Leukemia

Remission Induction

Initial experience with vincristine and prednisolone indicated that these agents could be effective in adult acute lymphoblastic leukemia, but the frequency with which remission was achieved was less than in children, and, in particular, remissions tended to be of shorter duration. Addition of other drugs such as daunorubicin to the regimen increases the response rate. Another regimen which avoids the use of anthracyclines but has a similar remission rate, comparatively well sustained, combines methotrexate with vincristine, L-asparaginase, and dexamethasone. This regimen produces comparatively less myelosuppression and, as originally described, included high dosage methotrexate during remission as an alternative to prophylactic cranial irradiation and intrathecal chemotherapy. The blood levels of methotrexate were sufficiently high to achieve

therapeutic concentrations of the drug in the cerebrospinal fluid. Other drug regimens are usually complex and toxic, but have the potential to produce more lasting remission.

Maintenance Therapy

Identical principles as discussed in the case of childhood acute leukemia apply to the disease in adults. Central nervous system prophylaxis and systemic maintenance therapy appear to be of value, in contradistinction to acute myeloid leukemia. Consolidation or intensification therapy and allogeneic bone marrow transplantation are therapeutic measures that have been administered in remission, but nonetheless appear less successful in producing sustained remissions as in the typical childhood form of disease.

Acute Myeloid Leukemia

Induction of Remission

A usually employed regimen against which the efficacy of new approaches is compared is a 7-day intravenous infusion of cytosine arabinoside 100 mg/m^2/day plus three daily injections of daunorubicin 45 mg/m^2/day. Important limitations are the cardiotoxic and myelosuppressive effects, which are more serious in the elderly; for this reason, reduction in the daunorubicin dose to 30 mg/m^2/day has been reported to produce an overall better outcome in patients over 60 years of age. Variations on the regimen, such as addition of thioguanine, an increase in the dose of cytosine arabinoside, substitution of doxorubicin for daunorubicin, or substitution of m-AMSA for anthracyclines, have been reported to produce comparable remission induction rates.

Severe myelosuppression during the two weeks following treatment is usual, and assessment of the marrow aspirate three weeks after commencement of treatment generally provides a useful guide to response. About 60% of cases collectively grouped under the title of acute myeloid leukemia attain meaningful remissions following treatment with this regimen. About two-thirds of these do so after one course. In the remaining one-third, remission is usually attained after a second course of treatment, which is less myelosuppressive as the cytosine arabinoside is administered for five days, and only two injections of daunorubicin are given. Persistence of leukemia after second course is usually taken as an indication of refractory disease, and alternative forms of treatment, such as high doses of cytosine arabinoside or other agents, are considered.

Maintenance Therapy

There has been unhappiness with the capacity of continuous administration of chemotherapeutic agents during remission to reduce the incidence of relapse, in comparison with the situation in childhood acute lymphoblastic leukemia. A variety of other regimens has also been employed, including one or more further courses of the successful induction regimen soon after the onset of remission, a course of intensive combination chemotherapy later in the phase of remission, and intermittent courses of moderately intense chemotherapy in which the agents are varied in a cyclical manner. None has met with general acceptance as a means for reducing the rate of recurrence of leukemia.

Bone marrow transplantation has been increasingly employed as a form of treatment for acute leukemia. Transplantation of bone marrow from a histocompatible donor after treatment of the patient with lethal myelosuppressive doses of antileukemic therapy can occasionally result in long-term remission in patients with overt disease. The most promising results to date are in children or young adults with acute myeloid leukemia in

first remission. There is a reduction in the incidence of relapse in comparison with similar subjects treated by other means, although the benefits from this are counterbalanced to an extent by occurrence of early deaths as a direct consequence of the toxicity of the procedure, or from death due to graft-versus-host disease. Influence on long-term outcome still remains to be clarified, but it is nonetheless clear that this form of therapy is of proven benefit only in younger patients (those <40 years old) because graft-versus-host disease become more severe with increasing age, and is restricted to subjects who have a histocompatible donor, thus limiting the procedure to the minority of patients with acute leukemia.

Autologous bone marrow transplantation represents an approach to treatment of patients with acute leukemia in partial or complete remission, in whom allogeneic bone marrow transplantation is not possible because a histocompatible donor is not available. In this form of therapy, bone marrow is aspirated from the patient in remission, and stored in the frozen state, either without further manipulation or after being subjected to procedures aimed at selectively reducing the content of leukemic cells. The patient is then exposed to doses of total body irradiation or to antileukemic agents that would normally be excessively myelotoxic, in the hope of killing residual leukemic cells.

Recovery of hemopoiesis after such treatment is then achieved by re-infusing the stored bone marrow to produce an autograft. Such an approach is still undergoing evaluation, but appears to prolong survival in certain poor prognosis forms of hematological neoplasia.

One of the reasons for the expenditure of such effort to treat the patient after induction of remission is that relapse occurs in the majority of patients managed by conventional appro- aches within two years, and treatment of relapsed disease is generally less effective in terms of the frequency of remission reinduction and in the duration of further remissions.

Supportive Care

There is general acceptance that combination chemotherapy of hematological malignancy, especially acute leukemia, is ideally managed by specialist units in view of the advantages this provides. Some of these are:

1. The most effective regimens are complex, and the choice of the appropriate combination for a given patient requires experience and judgement.
2. Duration of remission appears to depend in part on the degree of leukemic cytoreduction during remission induction, implying that more intensive therapy is overall the most effective. The degree of marrow suppression induced by many therapeutic regimens makes sophisticated support facilities essential.
   Such facilities include experienced physicians and nurses, expert microbiological and virological services, the availability of isolation reversebarrier nursing areas, and a cell separator to provide leukocyte and platelet support. The constant requirement for venous access has resulted in development of long-term indwelling intravenous catheters which are surgically implanted, and which require very careful surveillance in neutropenic patients, in particular, to prevent infectious complications of the venous access route.
3. Psychological and social support are essential during the prolonged periods of intensive treatment. Support systems become highly developed in specialist units. Mutual confidence leads to increased use of outpatient rather than inpatient care.
4. Cytotoxic drug dispensing requires specialized equipment and expertise. There are potential mutagenic risks for pharmacists and nursing staff from exposure to cytotoxic drugs, either through contact with skin or from inhalation of aerosol droplets containing

drugs. The wearing of gown, mask, and gloves, and the use of biohazard laminar flow equipment, is now routine in the preparation of cytotoxic formulations in specialized units.

5. The concentration of experienced personnel and patients in specialist units makes the development and assessment of new regimens of treatment possible, particularly when the specialist units within a country or region collaborate in the assessment of such new therapy.

Specific Aspects of Supportive Care

Rather frequent transfusion of red cell concentrate is usually required during remission induction because of the rapid fall in hemoglobin level that commonly occurs at this time with regimens employed for treatment of acute myeloid leukemia.

Prevention and control of infection is of utmost importance. When patients are severely neutropenic, a reduction in the degree of contact with external pathogens is attempted by confining patients to isolated areas. The cost and labor-intensive nature of these facilities in relation to their marginal advantages have resulted in a tendency to rely more on approaches such as reverse barrier nursing. Even this measure has not been shown to be particularly effective in preventing infections in leukemic patients, as many infections are derived from flora in the patient's gastrointestinal tract. Antimicrobial nasal spray and mouth rinse, plus oral administration of nystatin and/or ketoconazole, are generally employed as a means of reducing the load of pathogens in the gastrointestinal tract, and reduce local oropharyngeal infection. Administration of broad-spectrum antibacterial agents is of less certain benefit as a prophylactic measure, although some advantage has been claimed in reduction of opportunistic infection by treatment with cotrimoxazole in conventional dosages.

Infections are extremely common during treatment, especially during the latter part of the remission-induction phase, when the white cell count is very low. The major principle of treatment of established infection is prompt clinical and bacteriological diagnosis, and prompt antibiotic, antifungal, or antiviral therapy appropriate for the organism or organisms responsible for the infection. Onset of fever under such circumstances must be followed by an immediate search for a focus of infection, especially in the mouth, intravenous line, lungs, urinary tract, or skin. *Pseudomonas aeruginosa* and other gram-negative organisms as well as organisms that are not common pathogens can be the cause, especially in patients already treated with anitbiotics. Bacteremia and septicemia are common, and thus blood cultures should be performed and broad-spectrum antibiotics administered immediately via the intravenous route.

Suitable antibiotic regimes for febrile patients in whom the organism has not yet been isolated usually include a combination of an aminoglycoside plus a broad-spectrum penicillin or cephalosporin. The possibility of infection with yeast or fungus should be considered, especially in patients already treated with corticosteroids and antibiotics. Oral moniliasis can be treated with topical nystatin or amphotericin, but systemic yeast or fungal infection requires parenteral treatment, usually with amphotericin in view of its broad spectrum of activity and the low rate with which resistance to it develops. Patients with severe infection unresponsive to appropriate antibiotics should be treated with the addition of leukocyte transfusion.

Hemorrhage is a very common problem and a frequent cause of death. It is usually a consequence of thrombocytopenia, but a contributing factor can be intravascular coagulation promoted by sepsis or products of certain types of leukemic cells. Prophylactic

transfusions of platelet concentrates are usually given during the remission induction phase, as it has been shown that prevention of extreme thrombocytopenia is associated with fewer early deaths during treatment. An often employed threshold count, below which platelet transfusions are given, is 20×10^9/L. Such transfusions can ususally be administered every 2–3 days to maintain the platelet count above 20×10^9/L, but in the presence of infection, bleeding, or antiplatelet antibodies resulting from previous sensitization by allogeneic blood cells or platelets, the requirement is usually greater. Allo-antibodies to platelets can become sufficiently potent effectively to obliterate the benefit of transfusion of platelets from random donors, and under such circumstances selection of partially or completely histocompatible donors can yield platelets that are less damaged after transfusion.

Disseminated intravascular coagulation is a major factor contributing to increased bleeding in acute promyelocytic leukemia. Prophylactic therapy with heparin has been advocated as a means of suppressing the degree of intravascular coagulation, but is likely that death from hemorrhage is a consequence of the consumption of coagulation factors and platelets, as active replacement of missing factors reduces mortality from bleeding, which is a particular complication of the treatment of this particular category of acute leukemia.

Meningeal leukemia may be present initially or develop during hematological remission in all forms of acute leukemia. It is far more common in acute lymphocytic than acute myelocytic leukemia. Antileukemic agents in common use enter the cerebrospinal fluid in subtherapeutic concentrations when administered systematically in conventional doses, and for this reason about one-half of the episodes of meningeal leukemia used to occur while systemic disease was in remission in subjects who had not received prophylactic treatment of the central nervous system. The diagnosis is suggested by inappropriate headache or symptoms of central nervous system abnormalities, and may be accompanied by signs of raised intracranial pressure. Confirmation is dependent on examination of the cerebrospinal fluid for the presence of leukemic cells.

Treatment of meningeal leukemia is commonly performed by intrathecal instillation of methotrexate in dose of 10 mg/m^2 twice weekly for 2 weeks, and then at weekly intervals until the cerebrospinal fluid is free of leukemic cells. To avoid bone marrow suppression folinic acid is usually administered concurrently, 15 mg orally 6 hourly for 24 hours. The other commonly used agent is cytosine arabinoside in doses of 30 mg/m^2 at a comparable frequency of administration. Such frequency access to the cerebrospinal fluid is usually a problem, and repeated lumbar punctures can be avoided by inserting a subcutaneous reservoir under the scalp, attached to a catheter which delivers injected material into the third ventricle. Cranial irradiation as described as a prophylactic measure is also performed. It is very difficult to eradicate meningeal leukemia totally, and for this reason, intrathecal therapy is usually continued at monthly intervals on an ongoing basis.

MYELODYSPLASTIC DISORDERS

The myelodysplastic disorders are a heterogeneous group of leukemia-related conditions characterized by various combinations of anemia, neutropenia, and thrombocytopenia, usually with a normocellular or hypercellular bone marrow. They have several features in common with acute leukemia, but their clinical course tends to be more chronic, and there is a lesser degree of blast cell infiltration of the bone marrow (less than 30%). Transformation to an acute myeloid leukemia occurs in some cases. The French-American-British group has classified myelodysplastic disorders into five categories employing the basic criteria summarized in Table 12.7.

Table 12.7: Myelodysplastic disorders: FAB classification	
Type	*Bone marrow*
Refractory anemia (RA)	Blasts <5%
Refractory anemia with ring sideroblasts (RARS)	Blasts <5% ring sideroblasts >15%
Refractory anemia with excess of blasts (RAEB)	Blasts 5–20%
Refractory anemia with excess of blasts in transformation (RAEBT)	Blasts 20–30%
Chronic myelomonocytic leukemia (CMML)	Peripheral blood monocytes >1 × 10^9/L

Clinical Features

Most of the patients are over 50 years of age, and anemia, recurrent infections or infections that are difficult to eradicate, and hemorrhagic manifestations are the main clinical problems. Transformation to leukemia results in a clinical picture identical to that of de novo acute leukemia. A secondary form of myelodysplasia is occasionally encountered in persons of any age who have had treatment with radiotherapy, chemotheraphy, or a combination of the two.

Blood Picture

There is generally a mild to severe normocytic, or midly macrocytic, anemia. The red cells may be dimorphic, both hypochromic and normochromic cells being present, particularly and refractory anemia with ring sideroblasts. Other red cell abnormalities include basophilic stippling and the presence of nucleated red cells (often with dyserythropoietic changes). Neutropenia of variable degree is usually present. Sometimes, there is a shift to the left in the neutrophil series, and occasionally blast cells are seen. Hypogranular or agranular granulocytes, and granulocytes with bilobed Pelger–Huet nuclear configuration are commonly encountered. An increase in monocytes is characteristic of chronic myelomonocytic leukemia. Platelets are usually reduced, in some cases to very low levels, and are often dysfunctional.

Bone Marrow

The marrow aspirate is normocellular to hypercellular, and there is morphological evidence of disordered development of all cell series. The proportion of erythroid precursors varies considerably, but dyserythropoietic features are common and include asynchrony between maturation of cytoplasm and nucleus, megaloblastoid changes, multinuclearity, nuclear fragmentation, cytoplasmic vacuolation, basophilic stippling, and Howell–Jolly bodies. Marrow iron stores are usually increased, and ring sideroblasts are often present, being by definition very prominent in refractory sideroblastic anemia. Changes of dysgranulopoiesis include aberrant staining of the primary granules of myeloid precursors, hypogranular granulocytes, Pelger–Huet-type cells, and the presence of a variably increased proportion of blast cells. Micromegakaryocytes and large monolobular megakaryocytes are also seen.

Management approaches vary widely because these disorders not only vary widely in the extent of abnormal behavior within a particular category, but the different categories possess substantially different patterns of behavior. In many conditions, the clinical consequences can be sufficiently mild for no active therapeutic steps to be required. In situations where anemia is the only clinical problem of significance, transfusion alone may produce a good quality of life for many years, to the extent that transfusion-induced hemochromatosis can develop unless iron chelation therapy is administered to prevent toxicity from chronic iron overload. Refractory anemia with ring sideroblasts equates

with primary acquired refractory sideroblastic anemia, and is discussed in further detail in chapter related to iron deficiency anemia. Progression of chronic myelomonocytic leukemia can be suppressed or reversed by administration of relatively non-toxic agents such as mercaptopurine or hydroxyurea.

In refractory anemia with excess blasts, other major problems that can be encountered are infection and hemorrhage, due to inadequate numbers, or disordered function, of neutrophils and platelets. Transformation to overt acute myeloid leukemia occurs with moderate frequency in this disorder, and more frequently in refractory anemia with excess blasts in transformation. Response to treatment after transformation is less satisfactory even than in de novo actue myeloid leukemia. Sometimes, there is very substantial improvement in cytopenia, tantamount to remission of these disorders, following treatment with low doses of cytosine arabinoside for extended periods of up to three weeks, but responses to intensive treatment are generally less satisfactory than in the case of acute myeloid leukemia.

CHRONIC LEUKEMIAS

Usually seen are:
- Chronic myeloid leukemia, and
- Chronic lymphocytic leukemia.

CHRONIC GRANULOCYTIC LEUKEMIA (CML/CGL)

Chronic myelocytic leukemia, chronic myeloid leukemia and chronic myelogenous leukemia are synonyms for chronic granulocytic leukemia.

Clinical Features

Chronic myelocytic/granulocytic leukemia (CML/CGL) is a disease predominantly of middle life, the majority of cases occurring between the ages of 30 and 60 years, with a maximum incidence around the age of 45 years. It is rare under the age of 20. In older children, the disorder resembles that of adults, but a distinctive juvenile variety occurs in younger children. The sex incidence is approximately equal.

Onset is usually insidious, symptoms often having been present for many months before diagnosis. The majority of patients first seek medical advice because of symptoms due to anemia, splenic enlargement, or raised metabolic rate, either alone or in combination. Presenting manifestations are listed in Table 12.8.

Table 12.8: Presenting features of chronic granulocytic leukemia

- Common
- Anemia
- Splenomegaly
- Fatigue
- Weight loss
- Moderately common
- Night sweats
- Minor bruising
- Occasional
- Joint pain
- Bone pain
- Amenorrhoea
- Priapism
- Accidental discovery on routine blood examination

The most prominent symptoms are those of anemia—fatigue, weakness, pallor, and dyspnea.

Constitutional symptoms due to the raised metabolic rate are common in relatively advanced disease and include malaise, weight loss, and night sweats. Malaise, sometimes with marked exhaustion and prostration, is often a prominent symptom, especially in patients with high or rapidly rising white counts.

Symptoms resulting from *splenomegaly*, when it is marked, include a feeling of weight, dragging, or actual pain under the left costal margin, gastrointestinal symptoms, especially dypepsia, flatulence and easy satiety after eating, and swelling of the abdomen.

In some cases there is little or no gastrointestinal disturbance, despite marked splenomegaly. The accidental discovery of an enlarged spleen by the patient is sometimes the presenting manifestation. Acute pain over the spleen may occur following splenic infarction, and may mimic an acute abdominal emergency.

Hemorrhagic manifestations. Easy bruising, sometimes with the occurrence of hematomas, can occur, although severe bleeding is unusual.

Nonspecific skin lesions, herpes zoster, and leukemic infiltration are much less common than in chronic lymphocytic leukemia. Pruritus without any obvious skin changes occurs occasionally. Leukemic skin infiltration is of unfavourable prognostic significance.

Bone and joint pains occur occasionally, and the sternum is sometimes, but not usually, tender to pressure. Radiological changes in the bones are uncommon, but localized areas of cortical destruction, or less frequently of sclerosis, are occasionally seen. The blood uric acid is frequently raised, but gout is relatively uncommon.

Amenorrhea is a frequent complication in advanced disease, whilst menorrhagia may occur if the platelet count falls. Priapism due to obstruction to blood flow in the corpus cavernosum is an occasional but distressing complaint which is difficult to treat.

Infiltration of the nervous system is uncommon, and *nervous system* manifestations for the most part are due to hemorrhage. The nature of these manifestations depends on the site and the extent of the hemorrhage. Hemorrhage into the ocular fundus may cause impairment of vision, and hemorrhage into the internal ear, deafness and vertigo.

Fever is usually not a marked feature until the later stage of the disease, when it is common, but it is often absent in the early stages.

On examination at the time of diagnosis, splenomegaly is the outstanding physical sign, and apart from pallor and slight to moderate wasting, it is frequently the only abnormal finding. The spleen can be enlarged to below the level of the umbilicus. It sometimes extends into the left iliac fossa, and occasionally even to the right. It is firm and retains its normal contour, and the notch is easily felt. Following infarction, a rub may be felt or heard over the spleen. The spleen often becomes impalpable after treatment, and in rare cases it is not palpable at the time of diagnosis, for example when the condition is detected by routine blood examination. Smooth, moderate hepatomegaly is usual. *Lymph node enlargement* is much less common than in chronic lymphocytic leukemia.

The degree of *wasting* increases with progression of the disease. Bruising is sometimes present, but purpura is uncommon, as are hemorrhages into the fundus oculi.

Juvenile type: This rare disorder in children (usually under three years) bears certain resemblances to chronic granulocytic leukemia but tends to involve greater lymph node enlargement, less marked splenomegaly, more frequent infections, facial rash, and more hemorrhagic manifestations. The total leukocyte count is usually not as high, the total monocyte count is increased, thrombocytopenia is usual, and the disorder is Philadelphia-chromosome negative in contrast to the adult type. It tends to run a relatively shorter

course, with a worse response to chemotherapy than the adult type, and bone marrow transplantation, where possible, offers long-term remission and cure.

Blood Picture

The classical blood picture in chronic granulocytic leukemia at the time of presentation with clinical features of the disorder is of moderate anemia, and a markedly elevated total white cell count with a full spectrum of cells of the granulocyte series, including 20% or more of myelocytes. The platelet count can be normal, although it is raised in about one-half of cases, sometimes markedly (Fig. 12.4).

At the time of diagnosis, the anemia is usually of moderate degree, with hemoglobin levels from 8 to 10 g/dL. As the disease progresses, anemia becomes more severe. Red cells are usually normocytic and normochromic, and a small proportion of erythroblasts are occasionally present.

The leukocyte count ranges up to 500×10^9/L, or even higher. Segmented neutrophils and myelocytes constitute the majority of cells. Neutrophils vary in size, giant and dwarf forms being common. Myelocytes are the characteristic cells and comprise 10–50% of the white cells. The vast majority are neutrophilic, although a few are eosinophilic and basophilic. Myeloblasts comprise up to 10%, but can rapidly increase in proportion when 'blast crisis' occurs. An increase in the proportion of basophils (2–10%) is a characteristic feature and can increase further as the disorder progresses toward transformation to acute leukemia. The neutrophil alkaline phosphatase activity is markedly reduced.

Changes of only minor degree may be present when the disorder is detected by routine blood examination, as would be expected for the disease at a relatively early stage in its evolution.

The serum vitamin B_{12}, level and unsaturated vitamin B_{12} binding capacity are frequently increased.

Bone Marrow

Marrow aspiration yields hypercellular fragments with complete or partial replacement of fat spaces. The cell trails are hypercellular. The cells are mainly of the myeloid series, the myeloblasts are also increased. The differential count of myeloid cells is similar to that of cells in the blood, although there is further shift to the left. Erythropoiesis is normoblastic, but sometimes dyserythropoietic. The myeloid: erythroid ratio is increased, due mainly

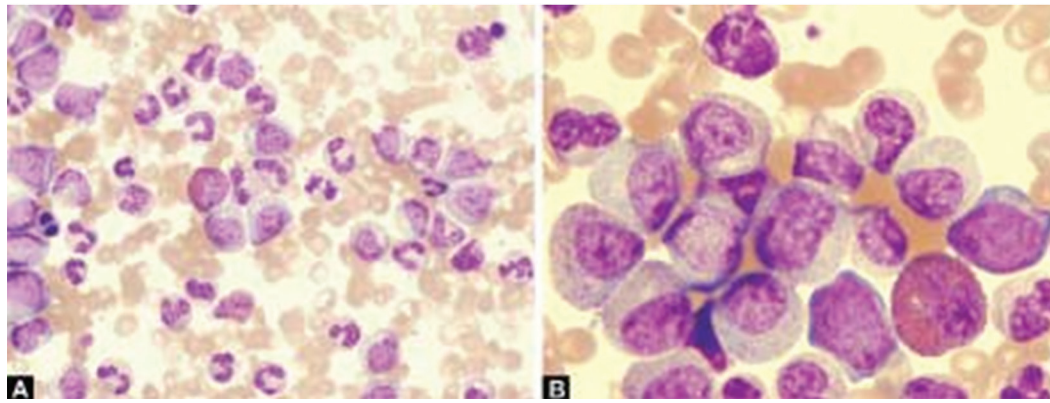


Fig. 12.4: CML, peripheral smears

to the white cell hyperplasia, but in the later stages there may be an actual reduction in erythropoietic tissue. Megakaryocytes are often prominent and are usually smaller than normal. Increased fibrosis is a notable feature of histological sections of the marrow in some cases.

Chromosomal findings: Cytogenetic examination of bone marrow and blood cells has shown that a specific abnormality, the Philadelphia chromosome, is characteristically associated with the neoplastic cells in chronic granulocytic leukemia. It is an abnormally small chromosome produced, as described previously, by reciprocal translocation between parts of the long arms of chromosomes 22 and 9 in nearly all instances. Following conventional treatment, the Philadelphia chromosome usually cannot be detected in the peripheral blood when the immature cells have disappeared, but it persists in the bone marrow cells. With the onset of blastic transformation, additional chromosome changes may develop. A relatively common occurrence is the appearance of additional Philadelphia chromosomes.

Course of Disorder

For most of its course, the disease behaves as a chronic process and responds predictably to therapy. The most frequent termination of the chronic phase is blastic transformation, where the characteristics of the disease usually alter radically to that of acute leukemia with a rapidly fatal course, usually with survival of less than six months. About 80% of patients with chronic granulocytic leukemia die as a result of blastic transformation, while the remainder die from intercurrent disease.

Prognosis: is difficult to predict in an individual case, as death, especially in younger subjects, is usually a consequence of blastic transformation, an event that occurs with a probability of about 25% per year. The average duration of survival from the time of detection is therefore 3–4 years. However, there is considerable individual variation in survival, from less than one year to 10 years or more.

Blastic transformation ('blast crisis'). Blastic transformation may be suspected clinically because it is commonly associated with symptoms of malaise, fatigue, anorexia, night sweats, bone pain, and splenic discomfort. The patient may feel markedly unwell before any overt changes appear in the peripheral blood or even in the bone marrow. Important physical signs are weight loss, pallor, pyrexia, sternal tenderness, progressively increasing splenomegaly, and the appearance of lymphadenopathy, which typically is absent in the chronic phase of the disease. Death usually results from hemorrhage or infection, or a combination of both, in disease unresponsive to treatment.

The most common change in the *peripheral blood* is a rapidly rising white cell count, appearance of a large proportion of blast cells, a falling hemoglobin level, and development of thrombocytopenia. The spectrum of white cell precursors characteristic of the chronic phase of the disease is lost, and a cell distribution typical of acute leukemia occurs, often with a predominance of blast cells and some segmented neutrophils. In most cases, the blast cells are myeloid in type, but in 20–30% the blast cells have lymphoblastic features. Examination of the bone marrow aspirate reveals an increased proportion of blast cells, which may constitute 80% or more of the cells.

Some cases of clinical deterioration are atypical in that the course is not a fulminating one, and blood examination may reveal no increase in immature cells. The bone marrow may appear unchanged or even hypoplastic, and a 'dry' or 'blood' tap is not uncommon. The diagnosis of this 'accelerated phase' of the disorder may be difficult to establish even in the presence of severe symptoms. Estimation of the neutrophil alkaline phosphatase level

may be of assistance, as it may rise after transformation has occurred. The development of new chromosome abnormalities may also be of diagnostic value.

Chloroma

Chloroma is a term used to describe the occurrence of localized subperiosteal leukemic tumor masses. The tumors are found more frequently in the skull, but they also occur in other bones, particularly the sternum, ribs, vertebrae, and sacrum. Chloroma develops both in acute myeloid leukemia and in chronic granulocytic leukemia, although in the latter it frequently denotes the onset of blastic transformation. The cells of the tumor masses usually contain sufficient porphyrin to give the tumor a green color, which rapidly fades on exposure to light. Tumor masses also occur in other organs and tissues.

Sometimes the tumors antedate the appearance of frank leukemic features by months or even a year or more. Temporary regression of tumor masses may occur after radiotherapy; otherwise, treatment is that of acute myeloid leukemia.

Differential diagnosis of chronic granulocytic leukemia must be made from conditions which can cause a similar type of blood picture. Of these, the most important are uncommon leukemic processes in which the Philadelphia chromosome is negative, and myelofibrosis, in which the clinical picture may be similar splenomegaly being the outstanding feature (Table 12.9). A 'leukemoid' blood picture associated with severe infection or secondary malignancy of bone may also cause difficulty when the spleen is palpable.

Treatment of Chronic Phase Disease

Unfortunately there is no curative treatment for chronic granulocytic leukemia, apart from allogeneic bone marrow transplantation. Treatment is usually palliative and symptomatic, the object being to effect the longest purposeful, and comfortable life for the patient. With adequate palliative treatment, it is usually possible to achieve not only a short increase of the actual time of survival but, more importantly, a significant lengthening of the comfortable and useful period of the patient's life. Thus, many patients who without treatment would spend most of their remaining life as chronic invalids are able to continue their normal occupations until a relatively short time before death occurs as a consequence of blastic transformation. The main form of palliative treatment is chemotherapy with busulphan. Splenic irradiation, which was the first effective form of treatment for this disease, has now been replaced by chemotherapy, as life expectancy has been shown to be superior with the latter treatment. The simplicity and ease of administration of chemotherapy, given adequate supervision, also argues strongly for this as standard treatment unless more effective eardicative measures are available.

Chemotherapy

Many of the chemotherapeutic agents cause temporary suppression of activity in chronic granulocytic leukemia. They include dibromomannitol, busulphan, thioguanine an hydroxyurea. Of these, busulphan causes control of a more predictable nature than the other agents, and the effects tend to last longer. Busulfan has therefore been the most often used chemotherapeutic agent.

Busulfan is sulfonic acid ester which is an orally absorbed alkylating agent, and is usually administered to adults in a dose of 4–6 mg per day. In general, there is progressive fall in white cell count, relief of symptoms, rise in hemoglobin level, and satisfactory control requires treatment for 2–4 months. treatment is relatively free from side-effects and does not cause nausea or vomiting. Neutropenia and thrombocytopenia are possible toxic

Table 12.9: Myelofibrosis vs. chronic granulocytic leukemia

	Myelofibrosis	*Chronic granulocytic leukemia*
Clinical features		
History	Possible preceding phase of polycythemia vera. Occasional history of splenomegaly for years.	
Splenomegaly	Usually marked	Moderate to marked
Fever	Uncommon	Common in uncontrolled advanced disease
Blood examination		
Anemia	Often slight to moderate despite marked splenic enlargement	Anemia usually appreciable when splenomegaly marked
Red cell morphology	Poikilocytosis with oval and tear-shaped cells prominent	Poikilocytosis not usually prominent
White cell count	Normal, raised, or low. When raised, seldom more than 50×10^9/L	Usually range 20–500×10^9/L
Nucleated red cells	Almost invariable and often numerous	Present in small numbers or absent
Neutrophil alkaline phosphatase	Normal, raised, or reduced	Reduced
Bone-marrow aspiration	'Dry' or 'blood' tap without marrow fragments usual Occasionally normocellular or hypocellular	Hyperplastic fragments with absence of fat spaces
Chromosomes	Philadelphia-chromosome negative	Philadelphia-chromosome positive
Bone-marrow trephine	Numerous fibroblasts. New bone formation common. Megakaryocytes often prominent. Collagen present as well as increased reticulin	Granulocytic hyperplastic replacement of fat spaces
Course	Chronic course over many years common	Chronic course unless blastic transformation occurs

effects, but are uncommon with the recommended dosage provided treatment is ceased at an appropriate time, such as when the leukocyte count has fallen 15–20×10^9/L. Blood examination should be performed every 1–2 weeks until satisfactory control is achieved, and then monthly.

When an essentially normal blood count is achieved, a decision must be made about whether to continue busulfan at a lower dose, or cease treatment until increased activity recurs, when a further course is given. Intermittent therapy is commonly employed. It is recommended that intermittent therapy be given when the doubling time of the leukocyte count exceeds 70 days, and continuous therapy when it is less. The dose of busulfan required for continuous therapy must be sought by trial and error, but is useful to begin with 2 mg daily, and to make adjustments according to the trend of the leukocyte count.

Side Effects

The main undesirable effect of busulphan is excessive myelosupprression, and this is more likely to occur if the initial dose exceeds 4 mg daily. Some patients are unusually sensitive to this dose, and there is a precipitous fall in white cell count. Treatment should

be immediately discontinued if this occurs, but it may be resumed later at a lower dose. Severe myelosuppression occurs more commonly in patients on continuous maintenance therapy.

Apart from bone marrow depression, side effects are few. The most common are skin pigmentation and amenorrhoea. More serious is a syndrome with features resembling Addison's disease. Pulmonary fibrosis can also occur. These side effects are more likely to develop with prolonged treatment. The syndrome resembling adrenal cortical insufficiency is characterized by weight loss, severe weakness, fatigue, anorexia, nausea, and pigmentation.

Other Forms of Treatment

Other chemotherapeutic agents that have been widely used with effective suppression of disease activity include dibromomannitol, hydroxyurea and thioguanine. Chlorambucil is also effective, but more likely to induce thrombocytopenia and immunosuppression.

Leukapheresis enables the leukocyte count to be lowered rapidly under conditions where vascular perfusion may be compromised by increased viscosity of the blood due to effects of extremely high leukocyte counts, a problem referred to as leukostasis. Effects of leukapheresis are transient, and this form of treatment is usually coupled with measures for suppressing proliferative activity of the disease.

Bone marrow transplantation has become increasingly employed form of treatment in younger subjects where a histocompatible bone marrow donor is available. This procedure can result in complete remission of chronic myeloid leukemia, an outcome not achieved by any routinely employed form of treatment with chemotherapy alone. Allogeneic bone marrow transplantation is associated with considerable immediate morbidity and mortality, but the threat to survival is less in fit young subjects than that of the almost inevitable transformation of the disorder to acute leukemia. Long-term survival following transplantation is now of order of 50%.

Treatment of Blast Crisis

Resistance to treatment with the drug that had previously been effective in the chronic phase of the disease is often one of the first indications of blastic transformation, and agents used to suppress chronic phase disease are generally unsatisfactory after blastic transformation has taken place. Combination chemotherapy is also less effective in inducing remission than in the corresponding forms of acute leukemia which have arisen de novo, although it is frequently possible to obtain remission of a relatively transient nature in the acute lymphoblastic variety of blastic transformation. Under the latter circumstance, reversion usually occurs to chronic phase disease, and it is under such conditions in particular that allogeneic bone marrow transplantation offers the prospect of 20% long-term remission in younger subjects, when a histocompatible donor is available.

CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

Classification of the Chronic Lymphoid Leukemias

B Cell

B-chronic Lymphocytic Leukemia

- Common type B-CLL
- B-CLL with >10% but 55% prolymphocytes (CLL/PLL)
- Richter's syndrome
  B-prolymphocytic leukemia (B-PLL) with >55% prolymphocytes.

Hairy Cell Leukemia

- Classic form (HCL)
- Variant form (HCL-V)

Plasma Cell Leukemia

- Lymphoplasmacytic
- Plasma cell and plasmablast

Leukemia Lymphoma Syndrome

- Splenic lymphoma with villous lymphocytes (SLVL)
- Waldenstrom's macroglobulinemia
- Follicular center cell
- Intermediate (diffuse centrocytic, mantle zone)
- Large cell

T Cell

- Large granular lymphocytic (LGL) leukemia
- T prolymphocytic leukemia (T-PLL)
- Adult T cell leukemia-lymphoma (ATLL, HTLV-1 positive)
- Sezary syndrome
- Peripheral T cell symptoms (HTLV-1 negative).

Clinical Features

Chronic lymphocytic leukemia (CLL) is a disease predominately of the middle and older age group, the majority of cases being detected between 45 and 75 years, with a maximum incidence at about 55 years. It is very uncommon under the age of 30 years. Males are affected twice as frequently as females.

The onset is characteristically insidious. Most patients present with enlargement of superficial lymph nodes, or with gradually increasing weakness and fatigue due to anemia, but not uncommonly the condition is accidentally discovered when the patient seeks medical advice for some other reason. Presenting manifestations are listed in Table 12.10.

Table 12.10: Clinical presentation of chronic lymphocytic leukemia

Common
- Lymph node enlargement
- Anemia
- Accidental discovery on clinical or hematological examination

Occasional
- Predisposition to infection
- Hemorrhagic manifestations
- Acquired hemolytic anemia
- Splenomegaly
- Gastrointestinal symptoms
- Skin infiltration
- Nervous system manifestations
- Bone or joint pains
- Mediastinal pressure or obstruction
- Disturbances of vision or hearing
- Tonsillar enlargement

Enlargement of the superficial lymph nodes is the outstanding clinical feature, with several, if not all, sites being involved, unless the disorder has been accidentally discovered by routine blood examination early in its course. The degree of enlargement varies. It is usually moderate but may be marked, especially in the later stages, when the nodes may exceed 5 cm in diameter.

The nodes are firm, discrete, not actually attached to the skin or superficial structures, and are usually painless, as in the case of the lymphomas. *Lymphadenopathy* can produce a variety of signs and symptoms, depending on the position of the enlarged glands. Such a form of presentation is similar to that in lymphomas except that the enlarged lymph nodes are usually more widespread when the patient presents.

Anemia invariably develops later in the course of the disease, and commonly anemic symptoms, such as slowly increasing weakness, fatigue, pallor, and effort dyspnea, are the presenting symptoms. However, anemia is not present in patients with early stage disease. An important complication in approximately 10% of cases is *acquired hemolytic anemia.* This is sometimes the first manifestation of chronic lymphatic leukemia. It should be suspected when the degree of anemia is inappropriately severe for the degree of lymph node and splenic enlargement, the degree of lymphocytosis, or when spherocytes or agglutination are present in the blood film. The importance is that it hemolytic anemia is not recognized, the patient may be thought to be in an advanced stage of the disease, and usually effective treatment for acquired hemolytic anemia will not be instituted.

Constitutional Symptoms

Due to raised metabolic rate, namely malaise, anorexia, fever, sweats, and weight loss, occasionally develop in advanced disease, but are usually absent for many months or years after diagnosis.

Splenomegaly: It is usually present at the time of diagnosis, and is usually less marked than in chronic myeloid leukemia, enlargement to below the umbilicus being uncommon. Nevertheless, the spleen is sometimes considerably enlarged and may extend into the left iliac fossa. In such cases, the patient may complain of a mass or of compression effects on the gastrointestinal tract. Mild to moderate *hepatomegaly* develops in most patients.

Purpura and other hemorrhagic manifestations: They usually occur in the later stages of the disease, but are uncommon at the onset. Occasionally the patient presents with spontaneous bleeding into the skin or with other hemorrhagic manifestations, such as persistent bleeding following trauma or tooth extraction. The cause of throm-bocytopenia can be a syndrome resembling idiopathic thrombocytopenic purpura, which tends to respond to corticosteroids or splenectomy in a similar manner to autoimmune thrombocytopenia. Thrombocytopenia can also develop as a consequence of impaired platelet production due to hemopoietic tissue replacement by the disease, or from myelosuppressive effects of agents used for therapy of the disorder.

Respiratory and other infections: Infections such as bronchitis and pneumonia are common in chronic lymphocytic leukemia, and infections at other sites are also increased in frequency, especially in advanced disease. The production of normal immunoglobin is often impaired, and this abnormality progresses through the course of the disease to result in increasing susceptibility to infection. Moderately reduced levels of IgG, IgA and IgM are relatively frequent findings. Neutropenia and corticosteroid administration are other factors that contribute to predisposition to infection, particularly to fungi and viruses, and infection is one of the most common direct or contributory causes of death.

Lesions of the skin: They are more common in chronic lymphatic than in chronic myeloid leukemia, and direct involvement may take the form of circumscribed, raised, brownish or purplered nodules of varying sizes, or of generalized infiltration with desquamation and thickening of the skin. There may be widespread erythema in patients with generalized infiltration by certain particular forms of lymphatic leukemia.

Tonsillar enlargement: It may occur, and occasionally enlargement of the lachrymal and salivary glands gives the picture of Mickulicz's syndrome.

Nervous system manifestations: They may result from infiltration of the nervous system, from pressure of enlarged node masses, or, in the later stages, from hemorrhage.

Blood Picture (Fig. 12.5)

The hemoglobin level ranges from normal values in virtually all cases of very early stage disease, through the moderate or severely depressed values in advanced chronic lymphocytic leukemia with extensive hemopoietic tissue replacement. Anemia is usually normochromic and normocytic under the latter conditions. When anemia is due to hemolysis, it usually has the typical features of autoantibody-mediated red cell destruction, with spherocytosis, a positive Coombs' test, and a reticulocytosis if the erythropoietic capability of the bone marrow is not impaired by infiltration of disease or by myelosuppressive effects of treatment.

The typical feature is a raised lymphocyte count, which at the time of diagnosis usually ranges from 50 to 200 × 10^9/L, although it is occasionally greater. When the disorder is detected on routine blood examination at an early stage in its development, the count can be appreciably less than 50 × 10^9/L, sometimes less than 10 × 10^9/L. Usually, 90% or more of the leukocytes are mature lymphocytes, mostly small with a thin rim of cytoplasm, although in some cases the lymphocytes are of medium size. The cells in the blood film tend to have a monotonous appearance, although some may be disrupted during the preparation of the film and are referred to as 'smear' cells. The absolute neutrophil count is usually in the normal range until the later stages of the disease, when it becomes depressed as a consequence of several factors, including replacement of normal hemopoietic tissue by the disorder, hypersplenic effects of an enlarged spleen, or myelosuppression by therapeutic agents.

In classical chronic lymphatic leukemia, the disorder is caused by monoclonal expansion of a B-lymphocyte population with a relatively low density of surface immunoglobulin. These cells have the relatively unusual and diagnostically useful property of forming rosettes with mouse, but not sheep, erythrocytes. There are a number of important variants in which the neoplastic lymphocytes are T cells. These conditions are considerably less common, except that one type occurs relatively frequently in certain geographic areas such as parts of Japan. Such disorders tend to pursue a more malignant course, be associated more commonly with infiltration of the skin, and respond differently to chemotherapeutic agents than the more common B-cell form of chronic lymphatic leukemia.

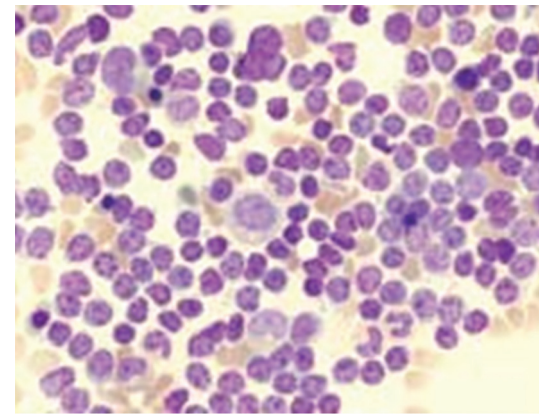


Fig. 12.5: CLL, peripheral smear

The platelet count initially is normal or moderately reduced, but, in the later stages, marked thrombocytopenia with counts of less than 50×10^9/L is common.

Bone Marrow

The classical findings in the bone marrow aspirate are an increase in lymphocytes and a corresponding reduction of megakaryocytes, myeloid precursors, and erythroid precursors, the extent of normal hemopoietic tissue replacement increasing as the disorder progresses.

Diagnosis

Diagnosis is usually simple. Detection of enlarged superficial lymph nodes or an enlarged spleen calls for a blood examination, which is essentially diagnostic.

Diagnostic problem can occur when there is a minor degree of absolute lymphocytosis but the white cell count is less than 10×10^9/L and the bone marrow is not extensively infiltrated with lymphocytes. Repeated examination over a long period may be necessary in such cases before a definite diagnosis can be made. The diagnosis may be overlooked in patients presenting with skin disorders, nervous system manifestations, and gastrointestinal symptoms, especially when superficial lymph node enlargement is not prominent. Differentiation generally have a greater density of surface immunoglobulin, and do not form rosettes with mouse erythocytes. Differentiation from states associated with reactive lymphocytosis can also occasionally be difficult, but the B cells under these circumstances form a polyclonal population, and the lymphocytosis often regresses with the passage of time.

Course and Prognosis

The average duration of life from time of diagnosis is 3–4 years, although individual survival varies from less than one year to ten years or more. Survival in general is greatly influenced by the stage to which the disease has advanced at diagnosis and by the rate of the progression.

Staging systems have been devised to provide an indication of prognosis. There are limitations in such a system, which for practical purposes is simple and cannot take every factor into consideration. For example, the influence on prognosis of enlargement of a single organ is not included, nor is the influence of immune versus non-immune cytopenia (particularly thrombocytopenia) taken into account.

Clinical Staging of CLL (Table 12.11)

Table 12.11: Clinical staging of CLL (old system)

		Median survival
Stage 0	Bone marrow and blood lymphocytosis only	>10 years
Stage I	Lymphocytosis with enlarged nodes	>8 years
Stage II	Lymphocytosis with enlarged spleen or liver or both	<7 years
Stage III	Lymphocytosis with anemia (Hb <11 g%)	2–5 years
Stage IV	Lymphocytosis with thrombocytopenia (Platelets <100,000 mm^3)	<2 years

Clinical Staging System Adopted by International Workshop on CLL

- **Stage A:** Lymphocytosis without anemia or thrombocytopenia and with fever with less than 3 areas of lymphoid involvement (includes stages O, I and II of the old system).
- **Stage B:** Lymphocytosis without anemia or thrombocytopenia but with more than 3 areas of lymphoid involvement (includes stages I and II of old system).
- **Stage C:** Lymphocytosis with anemia or thrombocytopenia or both (includes stages III and IV of the old system).

In fair number of patients, the condition causes little or no disability and may progress relatively slowly over many years. It is usually characterized initially by only slight to moderate lymph node and splenic enlargement, minimal or no anemia, and only a moderate lymphocytosis, e.g. 10–60 × 10^9/L, although exceptions occur. In such patients, it is not uncommon for the condition to be discovered accidentally, either when routine clinical examination reveals symptomless lymph node enlargement, or when blood examination reveals lymphocytosis. The disorder nonetheless progresses over potentially extremely long periods to cause increasing lymph node and splenic enlargement, anemia, neutropenia, and thrombocytopenia.

Many patients die from unrelated complications encountered in old age, but others die from consequences of bone marrow replacement by the disease, which causes anemia, neutropenia, and thrombocytopenia. Death usually results from cachexia and infection, often pneumonia.

Acquired hemolytic anemia or immune thrombocytopenia may develop at any point in the course of the disease, and, as treatable and reversible complications, should always be considered when anemia or thrombocytopenia is inappropriately severe for the stage of the disease.

Treatment

No therapeutic regimen for eradication of chronic lymphocytic leukemia is known. Treatment is consequently palliative and symptomatic, the object being to effect the longest possible active, useful, and comfortable life for the patient with the minimum amount of treatment. With optimum management, the patient can often lead a relatively normal life for many years. Approaches for suppression of disease activity include alkylating agents, corticosteroids, and radiotherapy. Splenectomy can be helpful especially in subjects with immune cytopenia, and parenteral γ-globulin in subjects with infectious problems due to hypogammaglobulinemia.

Radiotherapy

Radiotherapy is mainly used for treatment of an enlarged spleen or lymph node masses. It frequently produces substantial reduction in the volume of bulky tissues and can be useful for relieving obstructive effects, but even when radiotherapy is confined to one area, it may produce a surprising degree of myelosuppression in unirradiated sites, and thus should be employed with caution.

Chemotherapy

Chlorambucil is still considered to be the ideal chemotherapeutic agent for the treatment of chronic lymphocytic leukemia. It was found just over one-half of all patients treated for the first time obtained benefit, and most responded equally well to one or two further courses. The recommended initial daily dose was 0.15 mg/kg, or about 10 mg/day for a 70 kg adult. Blood counts should be performed weekly, and administration should be discontinued when the leukocyte count has returned to near normal levels. In most cases

it is necessary to discontinue the drug 4–6 weeks after starting treatment, and to allow 2–4 weeks for observation of the leukocyte counts, before resuming treatment at a lower dose of 5 mg/day or less, until a satisfactory response has been obtained. Response is manifested by a fall in lymphocyte count, sometimes followed by rise in hemoglobin level and platelet count, and regression in size of enlarged lymph nodes and spleen. Neutropenia is the most serious toxic effect, and treatment should be ceased if the neutrophil count falls below 1×10^9/L. Thrombocytopenia may also be induced, and the drug must be administered with caution if the patient has thrombocytopenia before treatment is commenced. It may be possible to withhold treatment for many months or even years after a satisfactory response to treatment has been obtained.

Cyclophosphamide can also cause regression of the disorder and is useful in patients with significant thrombocytopenia. The usual dose is 100–200 mg/day, and cross-resistance with chlorambucil is usually not encountered, so that cyclophosphamide may be effective in patients with disease refractory to chlorambucil. It does, however, have a tendency to cause neutropenia and therefore must be administered with caution.

Corticosteroids

On administration in sufficiently high doses, such as 25–100 mg prednisolone daily, there is often reduction in the activity of the disorder, with a decrease in size of lymph nodes and spleen, and a rise in hemoglobin level and platelet count. Beneficial effects can occur in disease refractory to chemotherapy or irradiation, as well as in combination with relatively lower doses of chlorambucil in patients in whom severe thrombocytopenia or neutropenia otherwise precludes treatment with alkylating agents. The dose of corticosteroid should be reduced as rapidly as possible because of the associated increase in susceptibility to infection. Sometimes, instances of the disorder refractory to chlorambucil alone achieve clinical benefit from a dose of predinsolone of approximately 10 mg/day, together with relatively low doses of chlorambucil.

Choice of Therapy

The therapeutic approach depends on the stage and activity of the disease, and whether auto-immune cytopenic complications are present.

Early stage chronic lymphocytic leukemia (stages 0-II): This stage requires no treatment. The patient should be encouraged to lead a normal life and undergo clinical and hematological assessment about every three months. Treatment is not required until signs of more active disease develop, which may not be for a number of years. This approach is being investigated by controlled trial of therapy in early disease, but there is no *evidence as yet to justify a change in attitude.*

Actively progressive, more advanced stage chronic lymphocytic leukemia: Anemia, systemic symptoms, pressure or obstruction from lymph node enlargement, and marked splenomegaly are indications for treatment. The choice between radiotherapy and chemotherapy is not always easy. Most commonly, treatment is instituted with chlorambucil, but radiotherapy can be considered when enlarged lymph nodes produce local compressive effects. Disease refractory to chlorambucil with or without prednisolone may benefit from combination chemotherapy with drugs including doxorubicin, cyclophosphamide, vincristine, and prednisolone, the former given in relatively low doses because of the high risk of myelosuppressive side effects.

Symptomatic and Supportive Therapy

Anemia is sometimes relieved by specific treatment of the disease, but in the later stages severe anemia is a common problem which responds relatively poorly to transfusion,

even when it is not caused by autoantibody-mediated hemolytic anemia. Corticosteroids sometimes produce relief, and occasionally surgical removal of an enlarged spleen results in reduction of transfusion requirements, although the procedure is accompanied by a high incidence of complications in such patients.

Patients in the later stages of the disease are more susceptible to infection, which should thus be treated promptly with appropriate antibiotics. Respiratory tract infections are especially common, and persistent chronic bronchitis may be particularly troublesome in the winter. In patients with recurrent infections, the administration of intravenous γ-globulin can be helpful in subjects with hypogammaglobulinemia.

Management of autoantibody-mediated hemolytic anemia or thrombocytopenia follows similar principles as for the idiopathic forms of these disorders, except that attempts are also made to suppress the activity of the leukemic process.

OTHER CHRONIC LYMPHOPROLIFERATIVE DISORDERS

Hairy Cell Leukemia (see Fig. 12.20)

Hairy cell leukemia is an uncommon disorder of middle and late adult life, characterized by the presence in bone marrow, spleen, and peripheral blood of abnormal mononuclear cells with hairy cytoplasmic projections, and best detected by phase-contrast microscopy. Males are affected more frequently than females. Most patients have marked splenomegaly, and lymphadenopathy is unusual.

The *blood picture* is often that of mild normocytic normochromic anemia with neutropenia and moderate thrombocytopenia. The proportion of hairy cells varies widely, but is usually in the order of 10–50%, and only occasional patients have a high leukocyte count with numerous hairy cells. Marrow aspiration is frequently difficult due to an increase in marrow reticulin, but in patients from whom a satisfactory specimen is obtained, extensive replacement of normal hemopoietic tissue with hairy cells is apparent. Infiltration is readily detected in trephine biopsies of bone marrow. The nature of the hairy cells varies, but most cases belong to the B-lymphocyte lineage.

Patients with minor degrees of cytopenia can be asymptomatic, and therapeutic intervention may be deferred if careful monitoring reveals an indolent clinical course. Clinical complications or overt progression of the disorder is regarded as an indication for intervention, especially the bleeding, infection, or anemia that occur commonly in advanced disease. Splenectomy is usually regarded as the treatment of first choice when splenomegaly is present, as splenectomy produces partial or even complete correction of cytopenia. It fails to produce any benefit in about 20% of cases. In the responders, the improvement is not sustained, although it may take many years for the cytopenias to recur as a result of bone marrow infiltration. Treatment at any stage of the illness with cytotoxic agents, such as chlorambucil in doses similar to those used in chronic lymphocytic leukemia, can sometimes produce substantial regression of the disease, and improvement in the degree of cytopenia, but with a high-risk of infectious complications.

More satisfactory responses have been obtained with long-term subcutaneous injections of *alpha interferon*, which produce marked and sustained regression of hairy cell leukemia, and reduce or eliminate its clinical manifestations in more than one-half of patients with advanced disease. The enzyme inhibitor *deoxycoformycin* also produces marked regression of the disease in a majority of patients so that prognosis in advanced disease has improved substantially in recent years due to the introduction of new therapeutic agents.

PROLYMPHOCYTIC LEUKEMIA (see Fig. 12.2P(a))

This is a rare form of lymphocytic leukemia in which the circulating lymphoid cells are larger and less mature in appearance than lymphocytes in chronic lymphocytic leukemia, but more mature than in acute lymphoblastic leukemia. It occurs more often in elderly males, and is associated with splenomegaly, but not particularly with lymphadenopathy. The prolymphocyte count in the peripheral blood can be greatly increased, as high as 200×10^9/L, and the abnormal cell population in most cases has the surface phenotype of B-cells.

The disease tends to progress more rapidly than typical chronic lymphocytic leukemia, and treatment is indicated at an early stage, although response to alkylating agents and corticosteroids is usually poor. Splenectomy, splenic irradiatioin, leucapheresis, and combination chemotherapy have been tried with varying and often little success in small numbers of patients, and the final place of these modalities in treatment is still uncertain.

LEUKEMOID REACTIONS

LEUKEMOID BLOOD PICTURE

The term *leukemoid reaction* is used to describe the occurrence of a peripheral blood picture resembling that of leukemia in a subject who does not have leukemia. The blood picture may suggest the presence of leukemia because of marked elevation of the total white cell count, or the presence of immature white cells, or both. Leukemoid reactions may be either myeloid or lymphoid. In general, a particular disorder causes only one type of reaction, but some may cause either a myeloid or a lymphoid leukemoid reaction.

Neutrophilic

Seen in—hemolytic crises, hemorrhage, Hodgkin's disease, infections, viz. tuberculosis; other bacterial infections, congenital syphilis, burns, eclampsia, mustard gas poisoning, vascular thrombosis and infarction, marrow replacement and myeloid metaplasia (Fig. 12.6).

Lymphocytic

Infectious lymphocytosis, infectious mononucleosis, pertussis, varicella, tuberculosis (Fig. 12.7).

Eosinophilic

Visceral larva migrans.

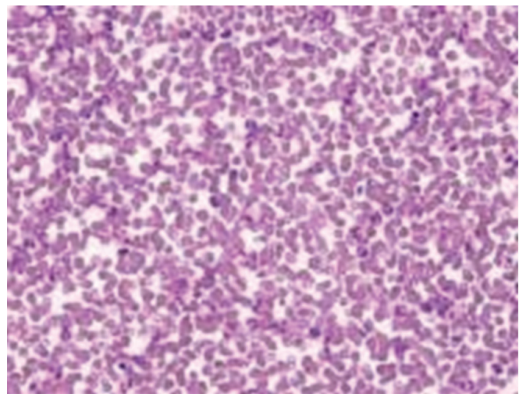


Fig. 12.6: Neutrophilic leukemoid reaction

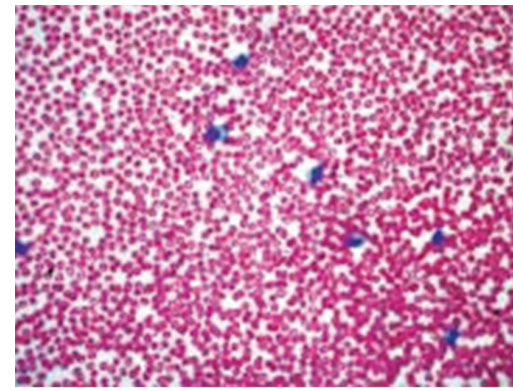


Fig. 12.7: Lymphocytic leukemoid reaction, infectious mononucleosis

DIAGNOSTIC PROBLEM

The disorders causing leukemoid blood reactions fall into two broad groups:

1. Conditions in which the blood picture suggests leukemia, but the clinical features of the underlying disorder causing the leukemoid reaction are obvious and suggest the correct diagnosis. The majority of cases fall into this group, and present little difficulty in diagnosis when both the clinical and hematological features are considered carefully.
2. Conditions in which both the blood picture and clinical features resemble leukemia, so that it is difficult to distinguish the leukemoid reaction from leukemia. These include cases in which there is splenic or lymph node enlargement, hemorrhagic manifestations, fever in the absence of any obvious infective process, and cases in which the percentage of immature cells in the blood is high.

Tables 12.12 and 12.13 enlist certain differences between leukemoid reactions and leukemia which help in differential diagnosis. However, in the individual cases, diagnosis can be difficult, especially when the underlying disorder causing the leukemoid reaction is not obvious. In such cases, further observation or investigation may reveal the true nature of the illness. Marrow aspiration and trephine biopsy are usually diagnostic, as the marrow changes in leukemoid reactions are seldom sufficiently marked to suggest leukemia. Cytogenetic studies may be helpful in the exceptional situation where an abnormal karyotype will establish that the process is of a neoplastic nature.

Table 12.12: Leukemoid reactions vs. Leukemia

	Leukemoid reactions	*Leukemia*
Clinical features	Clinical features of the causative disorder often obvious	Splenomegaly, lymph node enlargement, hemorrhage more common than with leukemoid reactions
Blood examination		
Total white cell count	Increase usually only moderate; seldom exceeds 100×10^9/L	Can exceed 100×10^9/L
Proportion of immature cells	Usually small or moderate. Myelocytes seldom exceed 5–15%, and 'blasts' 5%	Usually numerous
White cell morphology	Toxic changes may be seen in infective cases	Cells often atypical as well as immature. Toxic changes uncommon
Anemia	May occur, but often slight or absent	Usually present and progressive
Nucleated red cells	Frequent in leukoerythroblastic anemia due to marrow infiltration	Less frequent
Platelets	Mainly normal or increased, but reduced in leucoerythroblastic anemia and intravascular coagulation	Decreased, except in chronic granuloytic leukemia
Bone marrow	White cell hyperplasia may be present but seldom to same degree as in leukemia	Hyperplastic with potentially large proportion of immature cells
Autopsy	Infiltration of organs and tissues absent	Leukemic infiltration of organs and tissues

Table 12.13: Differential diagnosis between leukemoid reaction and CML		
	Leukemoid reaction	*CML*
Toxic vacuoles	2–4+	0–1+
Toxic granules	2–4+	0–1+
Dohle bodies	Frequent	Rare
Eosinophilia	0	1–3+
Basophilia	0	1–3+
PseudoPelger Huet	0–1+	Occasional
Karyorrhexis	0–1+	1–2+
Giant bizarre nuclei	1–1+	1–3+
Leukemic hiatus	0	Occasional
Neutrophil alkaline		
Phosphatase score	High	Low (most cases) Normal or high (rare)
Ph chromosome	—	+ (85% cases)

Myeloid Leukemoid Reactions

A myeloid leukemoid blood picture may be defined arbitrarily as one in which the total white count exceeds 50×10^9/L and myelocytes and/or myeloblasts appear in the peripheral blood. This is the usual type of myeloid leukemoid reaction, but occasional cases are seen in which immature granulocytes are present although the total white count is within normal limits.

CAUSES OF MYELOID LEUKEMOID REACTIONS

Infections

Leukemoid reactions due to infection are more common in children than in adults. With severe infections, the total leukocyte count may exceed 50×10^9/L, and a few myelocytes and promyelocytes, and even an occasional myeloblast, may be seen in the peripheral blood. Leukemoid reactions can occur, for example, following splenectomy when there is associated bleeding, hemolysis, or infection.

Most cases can be distinguished from leukemia on careful consideration of the clinical and hematological features:

a. The cause of the infection is usually obvious.
b. The percentage of the immature cells is small, e.g. 5–10%.
c. Anemia is slight or absent except in the presence of a complicating feature such as hemolysis or hemorrhage.
d. There may be toxic changes in the neutrophils as seen in infection and
e. The neutrophil alkaline phosphatase tends to be normal or increased in leukemoid reactions, which is useful in differentiating them from chronic granulocytic leukemia in which the value is subnormal.

Rare cases of disseminated tuberculosis simulating acute myeloid leukemia have been described.

Secondary to Nonhematological Malignancy

A moderate neutrophil leukocytosis of $15–30 \times 10^9$/L with a 'shift to the left' can occur in malignancy, especially with necrotic tumors or when there is complicating infection.

Occasionally, the white count exceeds 50 × 10^9/L, and a small percentage of myelocytes and myeloblasts are found. There is seldom any confusion with leukemia.

Acute Hemolysis

Leukocytosis with total white counts of 30 × 10^9/L or more, with the appearance of myelocytes in the blood, may occur in acute hemolytic anemia. Superficially, anemia, splenomegaly, and the presence of nucleated red cells may suggest leukemia, but appropriate investigation establishes the hemolytic nature of the disorder.

Leukoerythroblastic Blood Picture

The term leukoerythroblastic blood picture is used to describe the presence of immature myeloid and nucleated red cells in the peripheral blood, often as a consequence of disturbance of the bone marrow architecture by abnormal tissue.

Causes of a leukoerythroblastic picture are:

- Secondary carcinoma of bone; this is the most common cause
- Myelofibrosis
- Thalassemia major, especially after splenectomy
- Active hemolytic anemia
- Multiple myeloma (uncommon)
- Lymphoma (uncommon)
- Gaucher's and Niemann-Pick disease (rare)
- Marble bone disease (rare).

Characteristics of anemia associated with this abnormality vary somewhat with the disorder infiltrating the bone marrow, and are detailed in the appropriate sections. There are nonetheless certain general features. Anisocytosis and poikilocytosis (particularly tear-shaped cells are usual, and are often marked. A key feature is the presence of nucleated red cells, which are often numerous and disproportionately high compared with the number of reticulocytes (Fig. 12.8). They commonly number up to 10 or more per 100 white cells. A moderate increase in reticulocytes, e.g. from 3 to 10%, is usual.

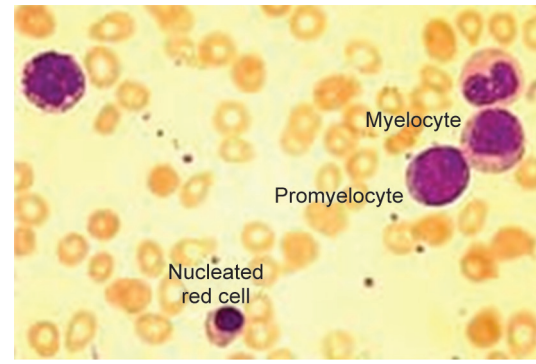


Fig. 12.8: Leukoerythroblastic blood picture

The white cell count is usually normal or moderately raised, but is sometimes reduced. The other key feature is that the leukocyte differential count shows a shift to the left, irrespective of the total white count, usually with the appearance of metamyelocytes, a few myelocytes, e.g. from 5 to 10%, and sometimes even an occasional myeloblast. The platelet count is normal or reduced, although it may be increased in myelofibrosis.

It should be remembered that immature red and white cells sometimes occur in the blood in disorders that are neither neoplastic nor diseases of bone, e.g. acute hemolytic anemias, megaloblastic anemias, and thalassemia major.

Causes of Lymphatic Leukemoid Reactions

Infectious mononucleosis and cytomegalovirus infection.

Pertussis may cause a marked absolute lymphocytosis which must be differentiated from chronic lymphocytic leukemia. In pertussis, the lymphocytes are usually normal and mature, and the hemoglobin level and platelet count are normal. Occasionally, the lymphocyte count in measles and chickenpox is high enough to simulate chronic lymphocytic leukemia.

Rare cases of tuberculosis with either a lymphocytic or lympoblastic blood picture have been reported. Most have occurred with disseminated tuberculosis, in which the lymph nodes, liver, and spleen were enlarged. Blood lymphocytes counts of over 50×10^9/L have been recorded. In the bone marrow, there can be a reduction in normal hemopoietic elements, either with or without an increase in lymphocytes.

Rare cases of carcinoma are associated with a marked rise in total lymphocyte count, e.g. to 20×10^9/L or more. The lymphocytes may all be mature, but sometimes a small proportion of lymphoblasts is present. In such cases, diagnosis from lymphocytic leukemia may be difficult in the absence of clinically obvious primary tumor.

Myelokathexis

Myelokathexis is a rare syndrome, possibly related to myelodysplasia but occurring in young patients and associated with chronic neutropenia and repeated infections. Marrow aspirates show many cells of the neutrophil series with hypersegmentation and larger than normal chromatin strands separating nuclear lobes. Binucleate myelocytes are a feature. The mature neutrophils are functionally defective.

CHAPTER

13

Paraproteinemias

Disorders characterized by abnormal proliferation of immunoglobulin producing cells and abnormal production of immunoglobulin represent part of the spectrum of disease due to neoplastic behavior of β-lymphocyte series.

CAUSES OF PARAPROTEINEMIAS

- Multiple myeloma
- Macroglobulinemia
- Malignant lymphoma
- Chronic lymphocytic lymphoma
- Benign monoclonal gammopathy
- Chronic cold hemagglutinin disease
- Rarely with carcinomas
- Heavy chain disease
- Primary amyloidosis.

CLASSIFICATION OF MONOCLONAL GAMMOPATHY

i. *Monoclonal gammopathy of uncertain significance* (MGUS)
   - Tumor-associated gammopathy
   - Biclonal gammopathy
   - Monoclonal gammopathy associated with miscellaneous disorders.

ii. *Malignant monoclonal gammopathy*
   - Multiple myeloma
     a. Smouldering myeloma
     b. Nonsecretory myeloma
     c. Plasma cell leukemia
   - Plasmacytomas
     a. Localized (solitary)
     b. Diffuse
     c. Extramedullary
   - Monoclonal gammopathy associated lymphoproliferative diseases.
     a. Malignant lymphoma
     b. Waldenstrom's macroglobulinemia
   - Heavy chain disease
   - Primary systemic amyloidosis

STRUCTURE OF THE IMMUNOGLOBULINS

The antibody molecules of normal human serum comprise five distinct classes of immunoglobulins which have been designated IgG, IgA, IgM, IgD and IgE. The basic unit of all immunoglobulin molecules consists of four polypeptide chains; two identical heavy chains (MW 60,000) and two identical light chains (MW 20,000). The heavy chains of IgG, IgA, IgM, IgD and IgE are referred to as gamma (γ), alpha (α), mu (μ), delta (δ), and epsilon (ε), respectively. Four subclasses of IgG, two subclasses of IgA, and two subclasses of IgM are recognized. There are two types of light chains, kappa (κ) and lambda (λ), each immunoglobulin molecule having either two kappa or two lambda light chains. Amino acid sequence analyses of light chains have shown that the amino-terminal half of the chain is characterized by a variable amino acid sequence (the variable region or V_L) and the carboxyl-terminal half by a constant sequence (the constant region or C_L). Heavy chains have a similar variable region (V_H) and three or four constant regions (C^1_H, C^2_H, C^3_H, and C^4_H). IgM occurs in serum as a pentamer of five linked IgM monomeric molecules, although small amounts of the monomer may be present. IgA is largely present as a monomer, but polymers also occur. There are two antigen binding sites on each immunoglobulin monomer, and theses are located at the apposed N-terminal regions of the light and heavy chains. The properties of the various immunoglobulins are summarized in Table 13.1, and The basic structure of the immunoglobulin molecule is depicted schematically in Fig. 13.1.

Table 13.1: Human immunoglobulins

	IgG	*IgA*	*IgM*	*IgD*	*IgE*
Heavy chain					
Class	γ	α	μ	δ	ε
Subclass	IgG_1, IgG_2, IgG_3, IgG_4	IgA_1, IgA_2	IgM_1, IgM_2		
Light chain					
Class	$\kappa_1\lambda$	$\kappa_1\lambda$	$\kappa_1\lambda$	$\kappa_1\lambda$	$\kappa_1\lambda$
Molecular formulae	$\gamma_2\kappa_2$	$(\alpha_2\kappa_2)_n$	$(\mu_2\kappa_2)_n$	$\delta_2\kappa_2$	$\varepsilon_2\kappa_2$
	γ_2	$(\alpha_2\lambda_2)_n$	$(\mu_2\lambda_2)_n$	$\delta_2\lambda_2$	$\varepsilon_2\lambda_2$
Molecular weight	160,000	170,000 × n	900,000	180,000	200,000
Sedimentation coefficient (S_{20},W)	7	7	19	7	8
Half-life in circulation (days)	21	6	5	3	2
Mean normal serum concentration (g/L)	12	2	1	0.03	0.0002
Intravascular fraction (%)	45	40	80	75	50
Complement fixation capability	+	0	+	0	0
Placental transfer	+	0	0	0	0

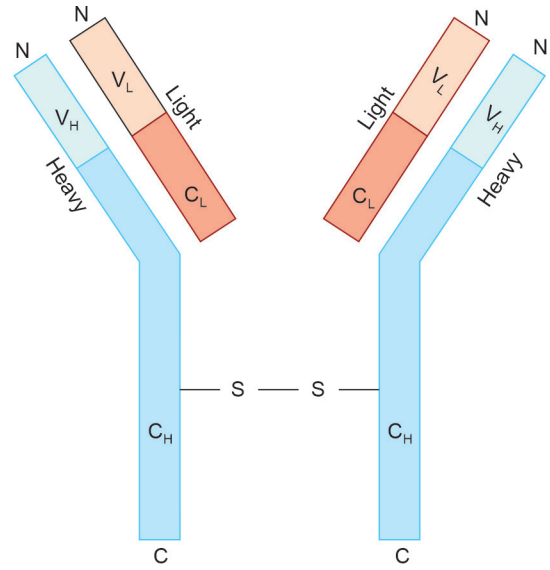


Fig. 13.1: Basic immunoglobulin molecule consists of two heavy and two light chains, linked by disulfide bonds. Each chain consists of a variable (V) and constant (C) region. N represents the amino-terminal amino acid of each polypeptide chain

Synthesis

Immunoglobulin is normally synthesized and incorporated into the outer membrane of resting B-lymphocytes. It is predominantly IgD or monomeric IgM, and serves as the means for recognition of specific antigen. Presentation of such an antigen to the B cell by the helper-inducer cell network results in cell proliferation and subsequent differentiation into a clone of plasma cells. These cells synthesize large amounts of antibody specific for the antigen, and secrete the antibody into the extracellular fluid.

The means by which cells are able to generate antibody to molecules to which the body has not been previously exposed is by rearrangement of segments of DNA in the genes that code for the individual chains of immunoglobulin molecules. The basic unrearranged 'germ-line' genes in a previously unexposed B-lymphocyte contain many individual segments, from which certain segments are selected, rearranged, and rejoined in such a manner as to produce a gene coding for an immunoglobulin with a binding site that is specific for the foreign antigen to which the cell has been exposed. The very large number of possible combinations of DNA segments involved in such a process is believed to account for the enormous diversity of different antigen-binding sites on antibodies which can be produced by the immune system.

Messenger RNA for the light and heavy chains is translated into protein by the abundant polyribosomes of the plasma cell. The chains are then linked together covalently and carbohydrate attached before the complete immunoglobulin molecule is secreted.

It is usual for more than one clone of plasma cells to be generated in response to exposure of the body to a foreign antigen, and the precise antigen-binding characteristics and the properties of the immunoglobulin molecule tend to vary from clone to clone. The net result of stimulation of the immune system by a particular foreign antigen is production of many similar, but not identical, antibody molecules directed against that antigen. About 70% of IgG antibodies to a particular antigen have κ, and 30% λ light chains. The polyclonal

nature of the immunoglobulins produced in the normal antibody response gives rise to the diffuse band in the γ-globulin region of the serum protein electrophoretogram.

Abnormal Synthesis

Abnormal synthesis of secreted immunoglobulin occurs when an abnormally large monoclonal population of plasmacytic or lymphocytic cells develops from a single precursor cell. This cell population produces identical molecules of immunoglobulin, or identical fragments of the immunoglobulin molecules. As the protein is homogeneous, the molecules migrate during serum electrophoresis as a discrete monoclonal (M) protein spike or band, and it is commonly referred to as a paraprotein. The presence of a paraprotein is one of the most common features of neoplastic plasma cell disorders, and also occurs in some instances of lymphocytic neoplastic activity.

Imbalance between light and heavy chain synthesis is common in such disorders. In myeloma, the most common abnormality is production of an excess of light over heavy chains. The excess light chains are secreted into the extracellular fluid, but pass readily through the glomerulus and are thus not retained in the circulation of intact immunoglobulin molecules. Light chains are catabolized by the renal tubular cells, but when present in excess pass into the urine as Bence-Jones protein. In about 25% of myelomas, only light chains are secreted by the neoplastic plasma cells, and this disorder is referred to as Bence–Jones myeloma. In heavy chain disease, fragments or intact molecules of the heavy chain are secreted by the neoplastic cells.

MULTIPLE MYELOMA

Multiple myeloma (can be solitary myeloma called plasmacytoma in extramedullary sites) is a neoplastic proliferation of plasma cells, characterized by lytic bone lesions, plasma cell (myeloma cell) accumulation in the bone marrow and the presence of monoclonal protein in serum and in about half the cases in urine also. It is a disease of middle and old age (50–70 years) and is uncommon below 40 years of age.

CLINICAL FEATURES AND COMPLICATIONS IN MULTIPLE MYELOMA (Fig. 13.2)

Bloodrelated Effects

- Cryoglobulinemia
- Hyperviscosity
- Bleeding disorders
- Interference with clotting factors and platelet function
- Raised serum globulins
- Hypoalbumenia
- Dilutional anemia
- Raised ESR, rouleaux formation

Tissue Effects

- Amyloidosis
- Organomegaly
- Proteinemia
- Renal failure

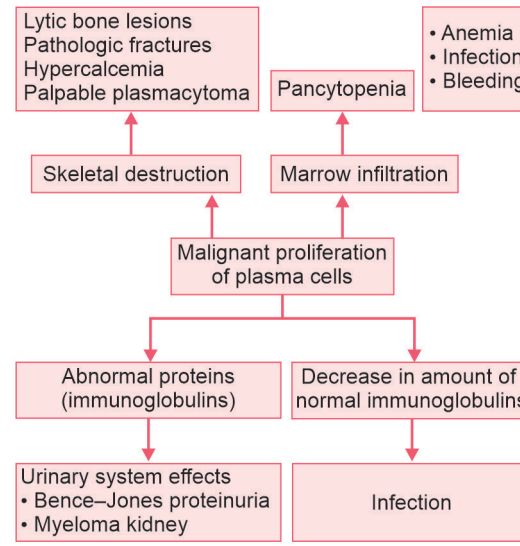


Fig. 13.2: Clinical features and complications in multiple myeloma

CLINICAL PRESENTATION

The majority of patients present with bone pain, symptoms of anemia, skeletal deformity, tumor formation, spontaneous fractures, or nervous system manifestations, either singly or in combination. Less common presenting manifestations are a bleeding tendency, renal insufficiency, and pulmonary infections.

Bone Pain

Bone pain is the presenting manifestation in about 70% of patients, and is usually the outstanding symptom. Nevertheless it may be absent, occasionally, throughout the whole course of the disease. Pain is most frequent in the lumbar, sacral, and thoracic spine, then in the rib cage, but it also occurs in the lips, legs, shoulders, and arms. It is common, and these problems are basically due to pathological fracture, lysis or compression.

Tumor Formation

Tumor formation is not uncommon and may occur on any bone, but especially on the ribs. Tumors vary in size, sometimes being quite large. They are generally firm and often tender. Occasionally, when the cortex is very thin, characteristic 'egg shell' cracking is felt and the tumors are fluctuant. The disorder sometimes presents with a single prominent tumor of bone, and occasionally the appearance of this apparently solitary growth precedes overt involvement of other bones by months or even years. In rare cases, there is a large single destructive tumor of bone, with the histological appearance of a plasmacytoma, which is cured by amputation or irradiation and is not followed by involvement of the skeleton elsewhere–solitary plasmacytoma of bone. Rarely, isolated extra-osseous plasmacytomas occur, and are more likely to be cured by excision or intensive irradiation than plasmacytomas involving bone.

Nervous System Involvement

Nervous system involvement is common. Most frequently it is due to compression by collapsed vertebrae or myeloma tissue. Compression of the spinal cord and nerve roots is the most frequent neurological complication, and may result in paraplegia or quadraplegia. Isolated peripheral neuropathy is a rare complication and its cause is unknown. Blunting of mental function occurs in the hyperviscosity syndrome. Disturbance of brain function ranging from drowsiness to seizures can be produced by hypercalcemia, and the development of hypercalcaemia must be suspected under such circumstances.

Anemia

Almost invariably occurs at some stage in the illness, and in advanced cases is frequently severe. It is relatively common at presentation, and an important contributing factor is the dilutional effect of large amounts of paraprotein in the circulation.

Renal Insufficiency

Chronic renal insufficiency frequently develops during the course of the disease, and occasionally is the initial manifestation. Retinal abnormalities are uncommon, and unless there is coexistent essential hypertension the blood pressure is normal. For this reason, myeloma should be considered as a possible cause in any patient with chronic renal insufficiency and a normal blood pressure.

The most important pathological changes in the kidney take the form of atrophy and dilatation of the tubules with cast formation in the tubular lumen. These are the

characteristic of 'myeloma' kidney. The casts are composed of a mixture of normal plasma proteins and precipitated Bence–Jones protein. Multinucleate epithelial cells are often found adjacent to the casts. Bence–Jones protein is also believed to damage renal failure include deposition of myeloma proteins, dehydration, local infection, hyperuricemia, hypercalcemia, and plasma cell infiltration of the kidney. Acute renal failure in the absence of previous renal impairment is less frequent, but may follow the hypovolemia and dehydration associated with some forms of intravenous pyelography. The Fanconi syndrome of renal tubular malabsorption can be associated with Bence–Jones proteinuria, and may precede the development of overt myeloma by several years.

Bleeding Manifestations

Bleeding manifestations occasionally bring the disorder to notice. Epistaxis and bleeding from the gums or into the skin are the most common bleeding manifestations, but melena, hematuria, retinal and other hemorrhages may occur. Several factors contribute to the pathogenesis of the bleeding, the most important being the presence of the abnormal protein. An effect on platelets by the paraprotein often leads to abnormal platelet function, with prolongation of the bleeding time and defective adhesion, aggregation, and platelet factor 3 availability. Thrombocytopenia may also contribute as it can occur in advanced disease.

The paraprotein may also act as an anticoagulant and inhibit some steps in the coagulation pathway. The most common abnormality is inhibition of fibrin monomer polymerization, which results in prolongation of the thrombin clotting time and defective clot retraction. Rare cases of interference with factor VIII activity have been described. Hyperviscosity also contributes to abnormal bleeding and bruising, but occurs more commonly in macroglobulinemia.

Infections

Infections are common in myeloma. They may be the presenting feature, and are an important cause of morbidity and mortality in myeloma. Chest infection with *Streptococcus pneumoniae* does occur, but infections with a wide variety of other gram-positive and gram-negative organisms are more common and involve virtually any part of the body. This predisposition to infection is multifactorial in nature. There are usually subnormal levels of normal immunoglobulins, and suppression of the normal antibody response, in particular the primary response. Neutrophils may function suboptimally, and their concentration in the blood is frequently depressed by interference with hemopoiesis due to marrow infiltration by myeloma, by cytotoxic chemotherapy, and even by the occasional development of myelodysplastic disorders later in the course of the illness.

Amyloidosis

Amyloidosis develops in up to 10% of patients. The distribution of the amyloid follows that of primary amyloid disease, in skin, heart, skeletal muscle, tongue, and gastrointestinal tract. Kidneys, liver, and spleen may also be involved. Renal involvement may lead to the nephrotic syndrome. The carpal tunnel syndrome is an occasional complication. The evidence that immunoglobulin light chains are the major protein component of amyloid fibrils in myeloma has been proven.

Visceral Involvement

Infiltration of the liver, spleen, lymph nodes, and other organs is commonly found at post-mortem, but rarely causes major clinical problems. The spleen and lymph nodes are

occasionally palpable, especially in cases of plasma cell leukemia. Moderate enlargement of the liver is more common.

Cryoglobulinemia

Five percent of myeloma proteins are cryoglobulins which reversibly gel in the cold and cause symptoms of cold intolerance. Cryoglobulinemia may precede the development of overt myeloma by several years.

Clinical Features in Relation to Immunoglobulin Class

The different immunochemical classes of myeloma have a tendency to have certain characteristic clinical features. IgG myeloma is associated with a higher level of paraprotein, a greater reduction of normal immunoglobulin levels, and more frequent infections than other types. Amyloidosis and hypercalcemia are less frequent. IgA myeloma is often complicated by hypercalcemia, and heavy Bence–Jones proteinuria is usual. Amyloidosis is not uncommon, but infection is less frequent. IgD myeloma is reputed to occur more commonly in younger patients, and hypercalcemia and renal failure are frequent. Heavy Bence–Jones proteinuria is usual. Amyloidosis and extraosseous tumor may occur. Bence-Jones myeloma also occurs in a slightly younger age group, and is particularly characterized by osteolytic lesions, hypercalcemia, renal failure, and amyloidosis.

BLOOD PICTURE

The blood picture in myeloma is not diagnostic. Nevertheless, the diagnosis is often suggested by the presence of certain features—marked red-cell rouleaux formation. The presence of atypical plasma cells, an abnormally blue-stained background in the blood film, and a greatly increased ESR.

Anemia is often present at diagnosis, or it develops during the course of the disease. The anemia is usually normochromic and normocytic in nature. A dilutional effect of the expanded plasma volume in patients with high concentrations of paraprotein contributes to the lowering of the hemoglobin concentration. Depression of erythropoiesis due to infiltration of the bone marrow, and the effects of cytotoxic chemotherapy are additional important factors. Other factors that can cause or contribute to anemia are renal failure, chronic infection, bleeding, and development of myelodysplastic or leukemic disorders.

The white cell count may be normal, raised, or moderately reduced, but moderate leukopenia is the most common, especially in advanced disease and in association with cytotoxic therapy. A leukoerythroblastic picture with the appearance of immature red cells and granulocytes develops in about 10% of patients. Small numbers of myeloma cells appear in the blood in about 20% of patients. Rarely, they appear in large numbers, when the condition is referred to as plasma cell leukemia. The platelet count is often reduced in myeloma.

The ESR is very often raised. Values frequently exceed 100 mm/hour and sometimes 150 mm/hour. However, the ESR can be normal or raised by only a moderate degree in those patients with Bence–Jones myeloma, in whom a paraprotein is absent from the blood. Blood grouping and cross-matching may be difficult because of red-cell rouleaux formation.

The clinical symptomatological differentiation of macroglobulinemia from multiple myeloma is presented in Table 13.2.

Table 13.2: Relative comparison of clinical features in multiple myeloma and macroglobulinemia		
	Macroglobulinemia	*Myeloma*
Organomegaly	+++	+
Hyperviscosity	+++	+
Lytic bone lesion	+	+++
Azotemia	+	+++
Length of survival	+ +	+

BONE MARROW (Fig. 13.3)

Bone marrow aspiration and trephine biopsy establish the diagnosis in most cases. Usually, infiltration with myeloma cells is diffuse, so that aspiration at any of the usual sites yields typical cells, but occasionally the lesions are focal, with areas of normal marrow between the tumor masses. In such cases, if the needle enters normal marrow the myeloma cells will be missed. Sometimes, diagnostic foci of myeloma cells are present in a trephine biopsy of bone marrow adjacent to a region from which the aspirate is not diagnostic. Biopsy of a radiologically abnormal or tender site is usually diagnostic, although sometimes a dry or blood tap is obtained when the aspiration needle enters a mass of myeloma tissue. If the overall features are suggestive of myeloma, a biopsy should be performed at an alternative site if the initial biopsy appearance is within normal limits.

The bone marrow fragments are usually hypercellular, and the cell trails usually contain myeloma cells. These cells commonly constitute 15–30% of the differential count, but higher percentages may occur. Myeloma cells vary in appearance from small, mature, differentiated cells resembling typical plasma cells, to large, immature, undifferentiated cells of 20-30 µm diameter. Many cells have intermediate characteristics. The cytoplasm of the mature cells is basophilic, sometimes with a perinuclear halo. The nucleus is commonly eccentric, and the chromatin is arranged in coarse strands, although it seldom shows the typical cartwheel arrangements which may be seen in the classical plasma cell. The cytoplasm of the more immature cells is abundant, light blue, and may show a perinuclear halo, vacuolation, and Russell bodies; the nucleus is more vesicular, with finer and evenly distributed chromatin. Nucleoli are common in the immature cells. Multinucleated cells are common, and mitoses are sometimes seen.

At postmortem in advanced disease, the marrow commonly has a grey gelatinous appearance, often with hemorrhage, and there is erosion and destruction of cortical bone

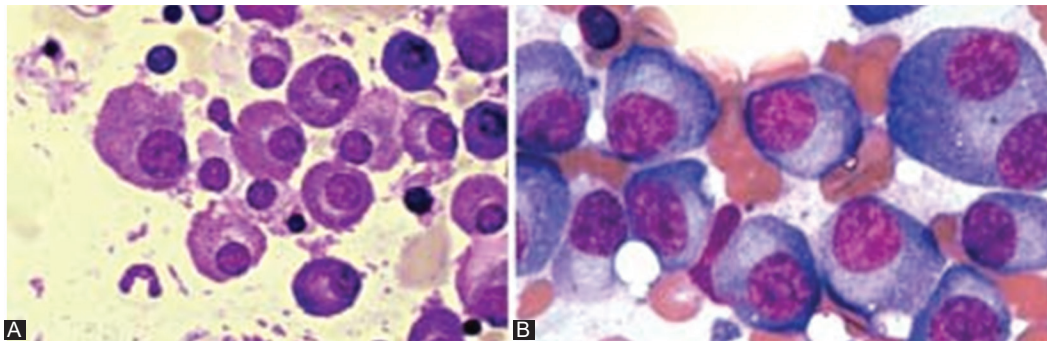


Fig. 13.3: Multiple myeloma, marrow

Increased proportions of plasma cell are present in the bone marrow in some other disorders. These include aplastic anemia, rheumatoid arthritis, hepatic cirrhosis, sarcoidosis, secondary carcinoma, systemic lupus erythematosus, and chronic inflammation. However, the plasma cells are usually mature and are seldom present in excess of 10%. In most cases, the diagnosis is obvious from the clinical and laboratory features. Occasionally, differentiation is more difficult, and diagnosis based on the appearance of individual plasma cells present in small numbers may not be reliable.

BLOOD CHEMISTRY

Total serum protein concentration is often increased due to the presence of paraprotein. However, a reduction in albumin concentration is common and tends to offset the effect of the increased amount of paraprotein. Total serum protein commonly ranges from 70–120 g/L, but may be higher.

The paraprotein usually appears as a single narrow homogeneous M-band on the serum electrophoretogram. Very rarely, there are two bands. Commonly, the concentration of normal γ-globulin is moderately to profoundly reduced. The position of the band on the strip varies from case to case. It is usually in the γ-globulin region, but occasionally is in the β-globulin region. An obvious paraprotein band is not evident in Bence-Jones myeloma or non-secretory myeloma, which make up 20–30% of all myelomas. In these instances, the serum protein electrophoretogram is normal or shows a reduction in γ-globulin.

Immunoelectrophoresis shows that the paraprotein is IgG in about 50% of cases, IgA in 25%, and IgD in 1%. From 50 to 70% of these patients have Bence–Jones proteinuria when tested for by sensitive techiques.

In the patients in whom there is no obvious M-band on the serum protein electrophoretic strip, Bence–Jones proteinuria is nearly always present, and free light chains can usually be identified in the serum by immunoelectrophoresis. These cases are referred to as Bence–Jones myeloma. In rare instances, in spite of clear-cut clinical and morphological evidence of myeloma, paraproteins or paraprotein fragments are not found in either serum or urine in the so-called nonsecretory myelomas. Other very rare types of myeloma are those associated with IgM and IgE paraproteins.

The serum calcium concentration is often raised. The concentration of phosphate is normal but rises when renal insufficiency develops. Serum alkaline phosphatase is normal or slightly raised, a point of importance in differentiation from hyperparathyroidism and secondary carcinoma of bone, in which significant elevation is usual. The serum uric acid concentration is often raised, even in the absence of renal insufficiency.

The plasma volume and serum viscosity are elevated in about 80% of patients. Viscosity does not usually reach a level sufficient to cause symptoms of the hyperviscosity syndrome, but hyperviscosity can occur in occasional cases of IgA myeloma and IgG myeloma of IgG1 and IgG3 subclasses.

URINE

Free monoclonal κ or λ light chains appear in the urine as Bence–Jones protein, and their detection is one of the bases of the diagnosis of myeloma. Bence–Jones protein was originally described in terms of its unique behavior on heating. Urine containing Bence–Jones protein flocculates when heated slowly to 50–60°C. The flocculated protein dissolves on boiling, and reappears on cooling below 60°C. Unfortunately, heat test is unreliable, especially with low concentrations of Bence–Jones protein. The most reliable means for the detection of Bence–Jones protein is electrophoresis of a concentrated urine specimen

on cellulose acetate. The monoclonal light chains usually migrate as a narrow band in the globulin region.

Electrophoretic methods detect Bence–Jones protein in the concentrated urine of 50–70% of myeloma patients with a serum paraprotein, and in nearly all patients who do not have a serum paraprotein.

More sensitive immunological techniques detect small amounts of free light chains in most normal urines, but these are polyclonal rather than monoclonal, and show broad electrophoretic mobility on electrophoresis. True Bence–Jones proteinuria is practically pathognomonic of myeloma, although it may occasionally occur in macroglobulinemia, amyloidosis, lymphoma, and leukemia.

BONE X-RAY

Bone X-ray changes occur in about 90% of patients at some stage in the course of the illness. Thus absence of bone change does not exclude myeloma. Bone changes consist of either diffuse decalcification or localized areas of bone destruction, or a combination of the two. The localized osteolytic lesions usually appear as multiple, rounded, discrete, punched-out areas with no sclerosis at the margin. They occur most frequently in bones normally containing red marrow, and are especially common in the skull. Diffuse osteoporosis is especially common in the spine, where wedge-shaped compression fractures are frequent.

DIAGNOSIS

a. In about 28% cases monoclonal protein occurs in serum and/or urine. Incidence of serum paraprotein IgG 66%, IgA 33%, IgM or IgD 1%

   The normal serum immunoglobulins are depressed. The Bence–Jones proteins found in urine are free light chains, either kappa or lambda, of the same type as serum paraprotein.

b. The bone marrow shows more than 10% plasma cells and often with abnormal—'myeloma' cells. A 60% of patients have osteolytic areas. A 20% show generalized bone rarefaction or osteoporosis which may cause pathological fractures. A 20% patients show no such lesions.

   Most often two of the three diagnostic features stated above are present. Given below are definitive diagnostic criteria.

Criteria for the Diagnosis of Multiple Myeloma

In order to qualify for the diagnosis of multiple myeloma a patient must satisfy one of the following combinations of criteria: la, and lb, la or lb and either 2a, 2b, 2c, or 2d.

1. *Cytologic criteria*
   a. Marrow morphology. Plasma cells and/or myeloma cells. In excess of 10 percent, when 1000 or more cells have been counted.
   b. Biopsy proven plasmacytoma, either in bone or soft tissue.
2. *Clinical and laboratory criteria*
   a. Myeloma protein (M-component) demonstrable by immunoelectrophoresis of urine.
   b. Myeloma protein (M-component) demonstrable by immunoelectrophoresis of plasma.
   c. Roentgenologic evidence of osteolytic lesions. Generalized osteoporosis qualifies as a criterion if the marrow contains in excess of 30% plasma or myeloma cells.
   d. Myeloma cells in at least two peripheral blood smears.

Other Laboratory Findings

1. Normochromic, normocytic anemia is usual. Marked rouleaux formation is seen. Neutropenia, thrombocytopenia seen in advanced cases. There may be myeloma cell spillover in the peripheral blood or there may be (very rare) plasma cell leukemia. Leukoerythroblastic changes (immature cells of both myeloid and erythroid series in the peripheral blood) are occasionally found.
2. About half the cases have raised serum calcium levels. Serum alkaline phosphatase rises secondarily to pathologic fractures.
3. ESR is markedly raised.
4. Renal damage leads to raised blood urea and serum creatinine levels.
5. Serum albumin is low.

The monoclonal peak (M peak) is found by using specific antisera and immunodiffusion techniques.

Emergency Situations

1. Uremia
2. Acute hypercalcemia
3. Compression paraplegia
4. Single painful skeletal lesion
5. Marked anemia
6. Bleeding diathesis.

Myeloma Staging System

Stage	Criteria	Measured Myeloma cell mass (cell × $10^{12}/m^2$)
I.	All of the following: 1. Hemoglobin value >10 g% 2. Serum calcium value normal (<12 mg/dL) 3. On roentgenogram, normal bone structure (scale 0) or solitary bone plasmacytoma only 4. Low M-component production rates a. Ig G value <5 g/dL b. Ig A value <3 g/dL c. Urine light chain M-component on electrophoresis <4 g/24 hours	< 0.6 low
II.	Fitting neither stage I nor III intermediate	0.6–1.2
III.	One or more of the following: 1. Hemoglobin value <8.5 mg/dL 2. Serum calcium value >12 mg% 3. Advanced lytic bone lesions 4. High M-component production rates a. Ig G value >7 g% b. Ig A value >5 g% c. Urine light chain M-component on electrophoresis >12 g/24 hours	>1.2 high

Subclassification

A = Relatively normal renal function (Serum creatinine <2.0 mg%)
B = Abnormal renal function (Serum creatinine value >2.0 mg%).

Example

Stage I A = low cell mass with normal renal function
Stage II B = high cell mass with abnormal renal function.

Management

Active therapeutic measures are warranted in progressive disease, and although treatment almost invariably does not eradicate myeloma, it can often suppress disease activity for many years and result in meaningful prolongation of life and relief of symptoms.

Symptomatic Measures

Some patients become very ill at some stage during the course of the illness because of hypercalcemia. Treatment by rehydration with intravenous fluids is particularly important, and often results in improvement of the reversible element of renal impairment produced by the hypercalcemia. Depression of the excessive plasma calcium concentration can be promoted by increasing the urinary excretion of calcium with certain diuretics such as frusemide, although other diuretics such as thiazides can have the opposite effect. Corticosteroids are particularly useful in suppressing the underlying osteolytic process, and doses of 50–100 mg prednisone or prednisolone daily often produce normal plasma calcium concentrations. The most important measure in obtaining sustained control of the hypercalcemia is suppression of the myelomatous process by chemotherapy.

Anemia may require correction by transfusion with packed red cells. Administration of oxymetholone has been shown in some studies to increase the level of hemoglobin.

Hyperviscosity can be corrected by plasmapheresis if this complication is causing clinical problems. Because circulating red blood cells contribute to blood viscosity, it is especially important to consider correction of hyperviscosity before blood transfusion, or to perform plasmapheresis during the transfusion.

Local pain, especially in bone, is a very common and serious problem in myeloma. Myeloma tissue is generally sensitive to radiotherapy, and as localized areas of bone pain are usually due to local infiltration, radiotherapy is often very helpful in producing a gradual reduction in pain and arresting the progression of the disorder in the irradiated area.

Nervous system compression is a relatively common problem, and localized compression effects by myeloma tissue may be reversed by radiotherapy, or by laminectomy where there is an urgent need to reduce pressure on the spinal cord.

Chemotherapy

Three agents have been used extensively in view of their ability to reverse the progression of myeloma when administered by the oral route, either singly or in combination. The most commonly employed drug is the alkylating agent melphalan (L-phenylalanine mustard). It can be administered continuously in a dose of 1–3 mg/day, but is more commonly given intermittently in a dose of 9 mg/m^2/day for 4 days every 4–6 weeks. Melphalan is myelotoxic, and as its absorption is variable the dosage must be adjusted either up or down according to the degree of myelosuppression indicated by serial monitoring of the blood count. Evidence that the proportion of patients who undergo an objective

response is almost doubled by concurrent administration of corticosteroid (e.g. prednisone 100 mg/m^2/day for 4 days).

Cyclophosphamide is about as effective in producing an objective response as melphalan. It does, however, tend to produce alopecia and chemical cystitis, but it can be effective in myeloma that is unresponsive to melphalan. It can be administered continuously in a dose of 1–3 mg/kg/day or intermittently in doses of 1000 mg/m^2 every 3 weeks.

High doses of corticosteroids such as prednisone or prednisolone 60 mg/m^2/day for 5 days, administered every 8th day for 3 courses, can produce objective responses as single-agent therapy, even in myeloma refractory to other drug treatment. Combinations including other agents are also employed. Additional drugs that are effective against myeloma include vincristine, Adriamycin, BCNU, CCNU and procarbazine. Most combinations include one or more of these drugs with corticosteroids plus melphalan and/or cyclophosphamide, and while they represent a more intensive chemotherapeutic attack on the disease they have yet to be unequivocally proven to produce a substantial increase in median survival over that obtained with melphalan and prednisolone. Interferon shows some promise in the treatment of myeloma.

Radiotherapy

Intensive radiotherapy may eradicate localized extraosseous plasmacytomas, but the usual wide-spread infiltration of the bone marrow in myeloma makes it difficult to utilize radiotherapy for treatment of the overall disease process.

Response and Prognosis

The eventual outcome in an individual patient with myeloma is profoundly influenced by a number of factors. There is an overall longer median survival in patients with stage I than stage III disease from the time of institution of chemotherapy. This, however, may reflect the earlier stage of progression of the disease process and the lack of osseous, hematological, and renal complications in stage I patients. Significant irreversible renal impairment is in particular associated with a poor prognosis. A dominant prognostic factor is the nature of the response to chemotherapy. Patients who obtain a very good response have a substantially longer median survival than those who are refractory to treatment. Objective responses to current treatment regimens occur in 50–70% of patients, and a considerable proportion of the responders live for many years longer than the median survival of about 18 months in untreated disease.

Although the extent of the tumor burden bears an inverse relation to survival, the outcome is nonetheless influenced by the rate of progression of the myelomatous process. In some untreated subjects in whom the parameters of the disease satisfy the diagnostic criteria of myeloma, the disorder may progress very slowly and exhibit a plateau-like phase of activity. Under such conditions, chemotherapy offers no significant benefit. Plateau-phase behavior is commonly observed after an objective response is achieved with chemotherapy, where the condition remains basically in a static, non-progressive state in the absence of further chemotherapy for up to many years.

The plateau phase of the disease is almost always terminated by recommencement of actively progressive myeloma. Such a development is associated with a poor prognosis when the patient is currently receiving chemotherapy, but further objective responses, even with the initially successful chemotherapeutic regimen, are relatively frequently attained when the patient has not been receiving chemotherapy during the plateau-phase. It has been argued that median survival is not increased by continuation of chemotherapy

during the established plateau phase in myeloma. The generally accepted approach is that administration of chemotherapy is required in either previously untreated or previously responsive disease when the disorder is undergoing active progression.

Progressive disease may be obvious from clinical manifestations such as worsening, osteolysis or marrow depression, but useful laboratory indices of progressive disease are progressive elevation of serum b_2 microglobulin and increasing levels of paraprotein in serum or urine.

Currently available chemotherapeutic regimes tend to reduce the bulk myeloma tissue and may substantially prolong life, but virtually never eliminate the disorder. Death from myeloma usually occurs from complications of progressive disease unresponsive to treatment, either at presentation or after recurrence of activity following an initial objective response. Common terminal events include complications of pancytopenia and immunosuppression, such as infection or hemorrhage, and complication of renal failure. An emerging, but less common, cause of death is the sequelae of myelodysplastic or acute leukemic disorder, which appear to be largely secondary to exposure to the alkylating agents used in the treatment of myeloma.

MACROGLOBULINEMIA

Macroglobulinemia refers to an elevation in the macroglobulin IgM in plasma or serum or both.

Classification of Macroglobulinemia

Benign

- Parasitic infections (trypanosomiasis, kala-azar)
- Rheumatoid arthritis
- Cirrhosis
- Cold agglutinin disease
- Tropical splenomegaly syndrome

Idiopathic

- Malignant
- Waldenstrom's macroglobulinemia
- Chronic lymphocytic leukemia
- Lymphomas
- Other malignancies

Waldenstrom's Macroglobulinemia

This is an uncommon disorder characterized by a neoplastic proliferation of cells of the B lymphocyte series, which produce large amounts of M paraproteins, called macroglobulins because of their high molecular weight. The pathoclinical features of the disorder are due to:

a. Proliferation of lymphocytic cells, causing marrow failure and enlargement of the liver, spleen and lymph nodes;
b. The physical presence of macroglobulins in the blood, which may increase the viscosity, interfere with normal hemostasis and gel at low temperatures (cryoglobulinemia).

Clinical Features

- Age group involved is usually from 50 to 70 years, males affected more commonly.
- Prominent symptoms are:

- Weakness
- Bleeding tendency
- Recurrent infections
- Visual disturbance

- Hepatosplenomegaly and lymph node enlargement seen in 50% of cases.
- Bone pain and tenderness are rare. Osteoporosis may occur.
- Peripheral neuropathy in some patients.
- Hyperviscosity syndrome. When the serum viscosity as measured by Ostwald Vascometer exceeds 4.0 (normal being 1.4–1.8) which usually corresponds to an IgM level of between 30 and 50 g/L, the characteristic signs and symptoms ensue:
  - Ocular changes
  - Mucous membrane bleeding
  - Neuropsychiatric manifestations
  - Congestive heart failure

Ocular symptoms range from minor blurring of vision to complete blindness. Fundal changes are striking, with stasis of blood in grossly distended and tortuous retinal veins, punctate hemorrhages, papilledema and rarely exudates. Recurrent epistaxes and bleeding from the mucous membrane of mouth and gums in the absence of thrombocytopenia is common. Neuropsychiatric manifestations include lassitude, headache, cerebellar dysfunction, confusion, coma and convulsions.

- Many patients develop an associated malignancy—the majority are lymphomas, but carcinomas may also *occur.*

Blood Picture

- Normocytic, normochromic anemia
- Red cell mass decreases and the plasma volume expands
- DLC is within normal range but mild lymphocytosis may occur
- Platelets are normal or decreased
- Outstanding feature is marked rouleaux formation in the peripheral blood and raised ESR often over 100 mm/hour (Westergren's method).

Bone Marrow

- There is marked increase in lymphocytes, a number of plasmacytoid lymphocytes are present
- Plasma cells may be increased
- Varying degrees of decrease in erythroid and myeloid cells and megakaryocytes are common
- Mast cells are often prominent

Lymph Nodes

The affected lymph nodes are moderately enlarged and show characteristic infiltration with lymphocytes and plasma cells, the reticulin pattern of the node being retoined. Some lymphocytes show PAS positive inclusions.

Blood Chemistry

- Total protein markedly increased, due mainly to increase in globulins which may vary from 20 to 120 g/L

- Cellulose acetate electrophoresis shows
  - Bence–Jone's proteins are present in small amounts in the urine of at least half the patients.
  - Normal immunoglobulins are not reduced to the same extent as in myeloma.

Treatment

- Plasmapharesis
- Chlorambucil and cyclophosphamide

HEAVY CHAIN DISEASES (HCD)

Malignant immunocytomas are characterized by excess amounts of heavy chains of a single class unattached to light chains. Three types corresponding to the three major immunoglobulins have been recognized:

- Ig G (γ-HCD)
- Ig A (α-HCD)
- Ig M (μ-HCD)

These are very rarely seen and diagnosed with difficulty.

BENIGN MONOCLONAL GAMMOPATHY (BENIGN PARAPROTEINEMIA)

- No malignant disorders are associated with them
- Prevalence rate is about 1% at the age of 50 years, increasing with advancing age.
- The majority of paraproteins are IgG, but there may be Ig A or Ig M. (with time the benign or malignant nature of the disorder can be ascertained, however, Bence–Jones proteinuria, subnormal levels of normal serum immunoglobulins, a paraprotein level in excess of 10 g/L and a progressive rise in its level point towards the diagnosis of malignant monoclonal gammopathy).

AMYLOIDOSIS

Paraproteins are found in urine and/or serum in nearly 90% of patients of primary amyloidosis. Such patients have no evidences of myeloma or macroglobulinemia. although organ distribution of amyloid resembles that of myeloma associated amyloidosis.

MALIGNANT LYMPHOMA AND CHRONIC LYMPHOCYTIC LEUKEMIA

The incidence varies from 5 to 10% of cases. This increased incidence is confined to diffuse lymphoma and chronic lymphocytic leukemia, the paraprotein being Ig M more frequently than IgG or IgA. Bence-Jones proteinuria may be detected in some patients. The incidence of paraproteins is not increased in Hodgkin's disease or nodular lymphoma.

MISCELLANEOUS DISORDERS

Paraproteins are occasionally identified in the sera of patients with:

- Hepatic cirrhosis
- Chronic infective illness
- Autoimmune disorders.

PRINCIPLES OF THE DIFFERENTIAL DIAGNOSIS OF MONOCLONAL GAMMOPATHIES

Evaluation of patients with suspected monoclonal garmmopathies involves the following principles:

Establish the Presence of a Monoclonal Gammopathy by Documentation of an M Spike

a. Perform serum protein electrophorosis.
b. Perform urine protein electrophoresis.
c. Perform immunoelectrophoresis to document the nature of the paraprotein.

Establish the Nature of Proliferating Cell Type

a. A bone marrow aspirate and biopsy will determine whether increased levels of lymphocytes, or plasma cells are present.
b. Perform a skeletal survey to determine whether the proliferating cells are focally aggregated or whether their activity is associated with bone lysis. Specifically, roentgenography is performed to determine whether there is demineralization of bone, i.e. diffuse osteoporosis or whether there are multiple or single areas of bone lysis.

Establish the Presence of an Entire or Partial Protein (Immunoglobulin) and the Activity of the Protein

a. Tests for Bence–Jones proteinuria (urine light chains)
b. Perform quantitative analysis of total immunoglobulins by radial immunodiffusion as well as quantitative analysis by immunoelectrophoresis.

Establish the Degree of Organ Involvement

a. Assess renal function (BUN, creatinine)
b. Investigate the skeletal system thoroughly
c. Evaluate the hemopoietic system

CAUSES OF HYPERVISCOSITY SYNDROME

Causes	*Diseases*
M-proteins	Waldenstrom's macroglobulinemia Multiple myeloma
Polycythemia	Polycythemia vera Severe secondary polycythemia
Leukocytosis	Chronic myeloid leukemia Other leukemias with very high TLCs
Hyperfibrinogenemia	Following factor VIII replacement therapy with large amounts of cryoprecipitate

CHAPTER

14

Lymphoid Tissue Tumors (Malignant Lymphomas)

Malignant lymphomas are broadly classified under two headings:
1. Hodgkin's disease
2. Non-Hodgkin's lymphomas

Although a number of classifications have been brought forward, yet the one given below is still most widely used.

CLASSIFICATION OF LYMPHOMAS HODGKIN'S DISEASE

Rye classification — Lukes et al. (Table 14.1)
- Lymphocytic predominance
- Nodular sclerosis

Table 14.1: Rye classification and brief description of various Hodgkin's lymphomas

	Pattern	*General age group*	*Description*	*Reed–Sternberg cells*	*Frequency*	*Prognosis*
1.	Lymphocyte predominant (LP)	Young individuals	Characterized by diffuse infiltrate of mature lymphocytes mixed with variable number of histiocytes	Few in number	5–10	Best
2.	Nodular sclerosis (NS)	Young individuals	Represents a distinct entity with a different epidemiology and biologic significance from the other three patterns. Unique to this pattern are two characteristics: • Presence of birefringent collagen bands traversing the lymph node and enclosing nodules of normal and abnormal lymphoid tissue; and • Presence of a RS cell variant called a lacunar cell, which looks like a Langhan's giant cell that sits in a lacunal space	RS variant (lacunar cells present)	35–60	Second best

Contd.

Table 14.1: Rye classification and brief description of various Hodgkin's lymphomas

	Pattern	*General age group*	*Description*	*Reed–Sternberg cells*	*Frequency*	*Prognosis*
3.	Mixed cellularity (MC)	Middle age	Characterized by a diffuse infiltrate of lymphocytes, histiocytes, eosinophils and plasma cells, which obliterate the underlying nodal architecture	Usually plentiful	30–50	Third best
4.	Lymphocyte depletion	Middle age	Characterized by a paucity of lymphocytes and diffuse fibrosis	Few in number	1–5	Worst

- Mixed cellularity
- Lymphocytic depletion
- Immunoblastic (B or T)

True histocytic tumors.

CLASSIFICATION OF NON-HODGKIN'S LYMPHOMAS

KIEL (LENNERT)

Low-grade Malignancy

- Lymphocytic (including CLL, hairy cells, and others)
- Lymphoplasmacytoid
- Centrocytic
- Centrocytic/centroblastic
  - Follicular
  - Follicular and diffuse
  - Diffuse
- Cutaneous T cell group
- T zone lymphoma

High-grade Malignancy

- Centroblastic
- Lymphoblastic [B, T (convoluted) or Burkitt types]
- Immunoblastic (B or T)

True histocytic tumors.

LUKES-COLLINS

Undefined

B-cell

- Small lymphocytic
- Plasmacytoid (lymphocytic)
- Follicular center cell (FCC) types nodular or diffuse
  - Small cleaved
  - Large cleaved

- Large non-cleaved
- Immunoblastic sarcoma

T-cell

- Small lymphocytic
- Convoluted lymphocytic
- Cerebriform (cutaneous)
- Lymphoepithelioid
- Immunoblastic sarcoma

Histiocytic

RAPPAPORT (MODIFIED)

Nodular and/or Diffuse

- Poorly differentiated
- Mixed
- Burkitt's
- Undifferentiated non-Burkitt's
- Histiocytic

Diffuse

- Well-differentiated lymphocytic (WDL)
- WDL with plasmacytoid differentiation
- Intermediate lymphocytic
- Immunoblastic
- Lymphoblastic
- 'Lennert' type
- Mycosis fungoides
- Plasmacytoma
- Unclassifiable
- Composite
- Malignant histiocytosis

NATIONAL CANCER INSTITUTE WORKING FORMULATION

Low-grade Malignancy

A. Small lymphocytic with or without plasmacytoid differentiation
B. Follicular, small cleaved
C. Follicular, mixed small cleaved and large cell.

Intermediate-grade Malignancy

A. Follicular, large cell
B. Diffuse, small cleaved
C. Diffuse, mixed small and large cell
D. Diffuse, large cell

High-grade Malignancy

A. Large cell immunoblastic
B. Lymphoblastic (convoluted a non-convoluted)
C. Small non-cleaved cell (Burkitt and non-Burkitt types)

Miscellaneous

- Composite
- Histiocytic
- Mycosis fungoides
- Hairy cell
- Unclassifiable
- Others

Lymph node enlargement is the most prominent feature of lymphomas. Table 14.2 depicts the various causes of lymph node enlargement.

Table 14.2: Lymphadenopathy–causes/sites

Anatomic site	*Neoplastic*	*Nonneoplastic*
Generalized enlargement	Leukemia Chronic lymphocytic acute, Chronic myelocytic (acute phase) Lymphoreticular neoplasms Hodgkin's disease Non-Hodgkin's lymphomas	Acute infections Bacterial, rickettsial, viral Chronic infections Tuberculosis, syphilis, toxoplasmosis Hypersensitivity states Serum sickness, drug reactions (hydantoin) Collagen-vascular disorders SLE, rheumatoid arthritis Endocrine metabolic disorders Hypothyroidism, hypoadrenocorticism. hypopituitarism
Cervical nodes	Lymphoreticular neoplasms Metastatic neoplasm • Nasopharynx • Oral cavity • Thyroid	Acute infections (as above) Chronic infections Tuberculosis, fungal Sarcoidosis
Supraclavicular nodes	Lymphoreticular neoplasms Metastatic neoplasms Bronchus, breast, stomach esophagus, pancreas, colon, genitalia Sarcoidosis	Acute infections (as above) Chronic infections Greater incidence reflecting intrathoracic tuberculosis fungal diseases
Axillary	Lymphoreticular neoplasms Metastatic neoplasms Breast, bronchus (rare)	Acute infections *Streptococcus, staphylococcus,* *P. tularensis,* cat-scratch disease
Mediastinal nodes	Lymphoreticular neoplasms Metastatic neoplasms Bronchus, breast, stomach, pancreas, colon, genitalia Sarcoidosis Erythema nodosum	Acute infections Pneumonia, lung abscess, viscus perforation Sporotrichosis Chronic infections Tuberculosis, fungal
Retroperitoneal nodes	Lymphoreticular neoplasms Metastatic neoplasms stomach, pancreas, colon, genitalia Tuberculosis, fungal	Acute infections *Salmonella,* viscus ulceration and/or perforation, abdominal Chronic infections
Inguinal nodes	Lymphoreticular neoplasms Metastatic neoplasms: genitalia rectum Syphilis, LGV, LGI	Acute infections *Streptococcus, staphylococcus* Venereal infections *Syphilis,* LGV, LGI
All sites	Congenital anomalies Lymphangloma, cystic hygroma	

The principal features differentiating Hodgkin's disease from non-Hodgkin's lymphoma are the presence of Reed–Sternberg giant-cells with their characteristic prominent nucleoli and the presence of variable numbers of lymphoid cells which are considered to be reactive to the disease rather than themselves being neoplastic. Hodgkin's tissue is also characterized by variable amounts of tissue necrosis and scarring and a tendency to spread to contiguous lymphoid tissue groups before distant hematogenous spread occurs. Non-Hodgkin's lymphomas are classified according to the primary cell type in the tumor, and also by whether the tumor is nodular or diffuse. There is little evidence of reaction by normal lymphocytes to the neoplastic process as judged by classical histological methods.

CLINICAL MANIFESTATIONS IN HODGKIN'S DISEASE

Common

Superficial lymph nodes enlargement

Less Common

- Fever, night sweats, weight loss and pruritus
- Pyrexia of unknown origin
- Mediastinal compression
- Abdominal pain
- Anemia, including hemolytic anemia
- Thrombocytopenia and leukopenia
- Features of nerve root and spinal cord compression
- Alcohol induced pain

Occasional

- Skin infiltration
- Nasopharyngeal obstruction
- Intestinal obstruction
- Malabsorption syndrome
- Bone pain
- Symptoms associated with hypercalcemia.

NON-HODGKIN'S LYMPHOMA (NHL)

Like Hodgkin's disease these tumors may arise at any age but show 2 peaks—(1) at childhood, and (2) from 50 years and onwards. Like Hodgkin's, males are affected twice more frequently than females. Broadly speaking the clinical presentation is more or less similar to Hodgkin's except for sites commonly involved. It shows less tendency to be confined to the axial lymph node structures than Hodgkin's at first presentation, and involvement of nasopharynx, tonsil and mesenteric structures is very much more common, as is involvement of the blood.

Lymph Node Involvement

- Enlargement may be gradual or tender, developing in weeks and may cause obstruction and pressure symptoms
- Extension outside the capsule induces pain
- The poorly differentiated NHL cause more local invasion and tissue destruction
- Involvement of epitrochlear lymph nodes is relatively common in nodular lymphocytic lymphomas.

Nasopharynx, Tonsil and GIT Involvement

- These sites are involved much more commonly as compared to Hodgkin's disease.
- In NHL (at autopsy) GI tract involvement is seen in 50–70% of cases. The most common sites being mesenteric lymph nodes, peritoneal deposits, hepatic involvement and involvement of small bowel.

Nasopharyngeal/Tonsillar Involvement Causes

- Soreness/pain in the throat
- Nasal obstruction or bleeding
- Lump in throat, dysphagia
- Regional nodal enlargement

GIT Involvement Causes

- GI bleeding
- Abdominal pain
- Vomiting or weight loss
- Steatorrhea (rarely)
- Intestinal obstruction

Massive enlargement of spleen is not uncommon and is frequently associated with coincident involvement of the liver. Extensive peritoneal deposits may cause ascites (usually chylous).

Systemic Features

- Are less common than Hodgkin's disease except in the presence of local skin involvement
- Pyrexia usually indicates secondary infection consequent on poor immune function.

(These patients commonly have hypogammaglobulinemia, cell mediated immunity may also be impaired.)

Autoimmune Disorders

These are more common in NHL and may give rise to acquired hemolytic anemia and thrombocytopenia.

DIAGNOSIS

This depends upon ruling out non-neoplastic causes of lymphadenopathy. Once these causes have been excluded, either on history or with relevant tests, or where doubt remains as to the cause, the next step is to establish a tissue diagnosis by means of biopsy. Wherever possible, this is performed on palpable superficial lymph nodes.

1. *Lymph node biopsy* is of utmost importance, it should be excised without trauma and properly fixed.
2. *Choice of node* as far as possible inguinal and upper deep cervical nodes should be avoided for these often show nonspecific inflammatory changes. Posterior triangle, supraclavicular and epitrochlear nodes are satisfactory because these are easily accessible and can be removed under LA. The largest available nodes should be excised.

Diagnosis when Superficial Nodes are not Enlarged

- Liver biopsy if liver involvement is suspected.
- Trephine biopsy of bone marrow if indicated.
- Scalene node biopsy if mediastinal involvement is suspected
- If necessary, diagnostic thoracotomy or laparotomy may be done.

STAGING

Stage

I. Nodal involvement within one region
II. Nodal involvement with two or more regions limited above or below the diaphragm
III. Nodal involvement both above and below the diaphragm
IV. Involvement of one or more extra-lymphatic structures

Each stage is further subdivided into:

A. No systemic symptoms
B. Documented fever, or loss of more than 10% body weight (Nodal involvement includes structures of Waldeyer's ring or spleen). Later modifications permit inclusion of I, II or III if a single extranodal site is involved, with suffix E followed by indentification of the site.

Staging Laparotomy

Where intensive radiation therapy is to be undertaken in the hope of achieving cure, laparotomy is the correct approach; it provides improved precision in evaluating the extent of disease and, in a significant number of cases, removal of a spleen containing unsuspected disease clearly justify this procedure.

FACTORS ADVERSELY INFLUENCING PROGNOSIS IN NON-HODGKIN'S LYMPHOMAS

1. Bone marrow involvement
2. Increased serum lactic dehydrogenase (LDH)
3. Presence of B symptoms, e.g. documented fever or loss of more than 10% body weight
4. Liver involvement
5. Presence of gastrointestinal mass disease
6. Hematocrit <36%; hemoglobin <12 g%

LYMPHADENOPATHY SIMULATING MALIGNANT LYMPHOMA

Follicular (Nodular) Pattern

- Nonspecific reactive follicular hyperplasia
- Secondary syphilis
- Rheumatoid arthritis (Felty's syndrome and Still's disease)
- Giant lymph node hyperplasia (hyaline vascular and plasma cell types)

Sinus Pattern

- Histiocytosis X
- Sinus histiocytosis with massive lymphadenopathy
- Lymphoma-like Kaposi's sarcoma
- Vascular transformation of sinuses
- Metastatic carcinoma and melanoma

Diffuse Pattern

- Post-vaccinial lymphadenitis
- Hydantoin (Dilantin) hypersensitivity
- Viral (Herpes zoster) lymphadenitis
- Immunoblastic lymphadenopathy
- Dermatopathic lymphadenopathy
- Lupus erythematosus

- Metastatic carcinoma and melanoma.
  Mixed Patterns
- Infectious mononucleosis
- Toxoplasmosis
- Cat-scratch disease
- Lymphogranuloma inguinale
- Metastatic carcinoma and melanoma.

PRETREATMENT CLINICAL EVALUATION OF A PATIENT WITH MALIGNANT LYMPHOMA

History

- Rate of onset
- Constitutional symptoms (anorexia, weight loss, fatigue, sweats, fever, pruritus)
- Symptoms of anemia
- Symptoms suggesting compression or obstruction by mediastinal, abdominal, axillary, pelvic and femoral lymph nodes
- Symptoms suggesting involvement of extranodal sites—nasopharynx, bone, GIT
- Symptoms of spinal cord involvement.

Examination

- Superficial lymph node enlargement—site and degree
- Splenomegaly and hepatomegaly
- Abdominal masses
- Signs of obstruction or pressure by mediastinal, abdominal, axillary, pelvic and femoral lymph nodes
- Signs of involvement of naso-pharynx. bone, or GIT
- Sign of spinal cord involvement
- Skin—rash, infiltration, herpes zoster, purpura
- Jaundice.

Special Investigations

- Lymph node biopsy
- Full blood examination, including platelet count
- Liver function tests
- Plasma urea and uric acid
- Serum protein electrophoresis
- Bone marrow aspiration and trephine
- Liver scan
- X-ray scan
- X-ray of lumbosacral spine and pelvis
- X-ray of nasopharynx
- Lymphangiography

Further Investigations Required in Some Cases

- Laryngoscopy or bronchoscopy
- Skeletal X-ray
- Intravenous or retrograde pyelography
- Venography (inferior vena cava)
- Barium meal or enema
- Immunoglobulin quantitation

TREATMENT

PRINCIPLES OF TREATMENT IN LYMPHOMA

General Considerations in Treatment

Advice to the Patient and Relatives

One must explain to the patient in clear terms, and the temptation to shrink from explaining the nature of the disease should be resisted other than under exceptional circumstances. If patients are to undertake a prolonged course of treatment requiring tolerance of side-effects and cooperation with drug therapy, they must be able to comprehend the reasons for the discomfort, and be motivated to seek recovery from the disease. Anxieties on the part of the patient with respect to family responsibilities and personal and financial worries may present real problems in management, and should be explored as far as possible in the early stages of the illness. Explanation to the patient and relatives should cover both the nature of the disease and the grounds for optimism based on the known results of therapy. The nature of sideeffects that are likely to be experienced should be discussed to allay anxiety when they arise, and to ensure the fullest cooperation when supportive measures are necessary. The patient should be encouraged to lead a normal active life within the limitations of treatment, and every attempt should be made to keep hospitalization to a minimum.

Therapy—its Objects

The fact that a significant proportion of patients with lymphoma may be cured means that careful assessment must be made in every patient, both with respect to histological diagnosis and extent of disease, so that the most appropriate therapy is undertaken. The basic choice falls between radiotherapy and chemotherapeutic approaches, but within the second of these alternatives a choice must be made between aggressive combination chemotherapy, with its promise of long-term remission and cure, and simple palliative approaches. The decision between radical treatment, palliative therapy, or observation with no active therapy in each case is one of the considerable importance and is influenced by assessment of the patient's capacity to tolerate radical treatment, given all appropriate supportive assistance.

Radical radiotherapy is the treatment of choice in localized Hodgkin's disease. It is very often curative in nonbulky disease which is localized to regions to which appropriately high doses of radiation can be delivered. Chemotherapy is, on the other hand, the treatment of choice for widespread or recurrent disease (Table 14.3).

In non-Hodgkin's lymphoma, chemotherapy is the mainstay of management, and radiotherapy has a limited curative role.

Table 14.3: Hodgkin's disease—treatment options

Pathological stage	*DXRT (Deep radiation therapy)*	*Chemotherapy (CT)*
IA and IIA	Upper or lower field (subtotal)	If there is bulky disease, e.g. mediastinal mass, CT may be added
IB and IIB	Total lymphoid	or CT
IIIA	Total lymphoid	or CT
IIIB and IV	—	CT

Radiation Therapy

The usual form of radiation is mega-voltage X-ray, providing least in the way of local and constitutional disturbances. Local X-ray therapy may be administered solely to involved regions, but generally treatment is planned to cover what is termed an upper or lower mantle field or both (total nodal irradiation). Only one mantle field is treated in a single course of therapy. An upper mantle field represents irradiation above the diaphragm with shielding to the lungs and much of the heart; it includes the deep and superficial lymphatic chains in the neck, supraclavicular and axillary regions, and mediastinal structures. It may be extended upwards to include Waldeyer's ring where appropriate, and down to the level of L4 to include the upper para-aortic nodes, and also to include the spleen if staging laparotomy is not performed.

A lower mantle field has the shape of an inverted Y, with shielding covering the lateral structures such as liver and kidneys, and the midline pelvic structures and gonads. During the course of therapy, patients ordinarily develop inflammation affecting the pharynx and oesophagus with upper mantle treatment, as well as some discomfort to the skin at the front and back. In lower mantle therapy, marked gastrointestinal disturbance is common. An acute radiation pneumonitis affects 5–10% of patients subjected to upper mantle radiotherapy, and a course of corticosteroids may be required to suppress the inflammation. General radiation sickness may occur, but is modified by the administration of antiemetic therapy. Typically, older patients suffer a greater degree of systemic upset with radiation therapy than the young, and this may rarely influence the choice or extent of treatment in individual patients.

Radical radiation therapy of this kind (ordinarily to a dose 35–40 Gy) invariably suppresses bone marrow proliferation in the irradiated area, and sometimes this necessitates temporary cessation of treatment. A rest period is usually required between upper and lower mantle treatment to allow hemopoietic recovery to occur, and should there be evidence during this period of spread of disease to sites not amenable to radiation, the treatment plan should be reconsidered and chemotherapy undertaken before further bone marrow damage results from radiotherapy. Rapid spread of this kind, however, is uncommon.

In some cases, it is appropriate to administer both radical radiotherapy and combination chemotherapy as initial treatment. The combined approach offers a better chance of cure in stage II Hodgkin's disease with a bulky tumor (e.g. mediastinal). The treatment may be administered by alternating several courses of chemotherapy with radiotherapy.

However, the combination of both modes of treatment increases the risk of a second tumor developing in the future, especially acute leukemia.

Response to Radiation Therapy

Hodgkin's disease is a highly radiosensitive tumor, and long-term survival rates relate very much to the extent of disease when treatment is first undertaken. Results have improved enormously in the past 30 years. The proportion of patients alive 5 years after diagnosis improved from 34 to 87%. In patients with disease of stage IA and IIA on presentation (patients without systemic symptoms and disease confined to treatable areas on one side of the diaphragm), figures relating to recurrence and survival at 5 years show a high probability of cure in the great majority of cases. Projected 10-year survival was greater than 80%, particularly where initial assessment included staging laparotomy to pick up the significant number in whom otherwise undetectable splenic disease would have been missed. Results of treatment in stage IB, IIB and IIIB disease are less satisfactory,

and practice varies in different centres as to whether such patients receive radiation or chemotherapy. Results in stage IIIB disease are little different from stage IV, making it unsuitable for radiotherapy, whilst stage IIIA, particularly if of a favorable histological subgroup, may be effectively treated by radiation.

Non-Hodgkin's lymphoma is also highly radiosensitive, but the rate of recurrence following radiation treatment varies considerably with the histological type, and can be much greater than in Hodgkin's disease. Lymphocytic types have a rapid response but have a tendency to recur in untreated regions. Histiocytic or poorly differentiated types may respond rapidly but high radiation dosages are required to prevent recurrence in treated areas.

Chemotherapy

Like chemotherapy of the infectious diseases, chemotherapy in neoplastic diseases is based upon the principle of selective toxicity. The ideal antineoplastic drug would be one with no action upon the normal body tissues but with a powerful toxic action upon the tumor. However, it is far more difficult to find an ideal antineoplastic drug than it is to find a suitably selective antibiotic, for, whereas bacterial cells are foreign to the body and have metabolic processes differing profoundly from those of human cells, the cells of malignant tumors are not truly foreign, and the metabolic differences so far detected are, in the main, quantitative. Consequently there is no ideal antineoplastic agent known. All the agents at present in use inflict damage of varying degree upon normal body tissues. As would be expected, the normal tissues most likely to be affected are those which proliferate more actively—the bone marrow, gonadal tissue, the epithelium of the alimentary tract, and the fetus, although exceptions occur. The agent of choice for a given neoplastic disease is the one that has the highest ratio of therapeutic to toxic effects, i.e. the greatest target specificity.

Responses to given agents vary greatly from one type of tumor to another. Even with a single type of tumor there are individual variations in degree of response from one patient to another; also the susceptibility to toxic side effects varies from patient to patient.

In order to gain additive antitumor effects and to minimize side effects, effective cytotoxic drugs are generally given in combination in such a way that they have as few additive side effects as possible on normal tissues, although bone marrow suppression is the usual dose-limiting factor.

Basic Principles for Use of Chemotherapeutic Agents

Cytotoxic drugs were initially used as palliative therapy in patients with advanced disease, but the introduction of aggressive combination chemotherapy for patients with Hodgkin's disease of stages III and IV revolutionized the approach to drug treatment of lymphomas. Not only may the disease be arrested, but cures are achieved in a significant number of patients hitherto facing inevitable death with their disease. Chemotherapy provides a varying, and sometimes considerable, meaningful prolongation of life in patients who have no chance of cure by radiotherapy, and is in fact becoming the most frequent means by which cure is attained in neoplastic disorders of this type.

Use of cytotoxic drugs in combination requires knowledge of the pharmacodynamics, effects, and side effects of each drug, and experience with the effects of the drugs used in combination. The experience of others is available to a considerable extent when a protocol is employed, the dosage and timing of drug administration being laid down in a manner which, from previous extensive experience, has been found to be safe and predictable. Many such protocols contain instructions concerning modification of dosage required in

the event of the development of bone marrow depression or other side effects, but in all forms of chemotherapy, close clinical and hematological supervision are essential. Careful documentation of response of the tumor and the occurrence of side effects, especially changes in the red cell, white cell, and platelet counts must be maintained throughout the period of treatment.

The occurrence of side effects of drugs is variable. Bone marrow is particularly susceptible to depression for months following intensive irradiation, or for some weeks following previous chemotherapy in patients with infiltration of the bone marrow.

Many side effects of drugs are potentiated by renal or hepatic failure, and are more prominent in the elderly, requiring diminution of dosage or sometimes the withholding of particular drugs when side effects become troublesome.

Palliative chemotherapy may be undertaken as the appropriate course in a patient unable or unwilling to tolerate more rigorous combination chemotherapy. In such instances, a single drug effective by the oral route, or combinations of drugs in doses less than those employed in intensive treatment, may be used. Such an approach may be appropriate in patients over the age of 70 years, but it must be remembered that cumulative side effects may still arise, depending on the drug used, and careful monitoring is still necessary.

Agents Used in Treatment of Lymphomas

Many chemotherapeutic agents are now available. The principal drugs may be grouped under the headings of alkylating agents (e.g. nitrogen mustard, cyclophosphamide, chlorambucil, nitrosoureas), vinca alkaloids (vincristine and vinblastine), procarbazine and dacarbazine (agents with actions similar to the alkylating agents), antibiotics with antitumor effects (e.g. Adriamycin and bleomycin), the folate antagonist methotrexate, and corticosteroids. Only those currently most widely used are discussed below.

Nitrogen Mustard

Nitrogen mustards are nitrogen analogues of sulphur mustard (mustard gas), a vesicant gas used in the first world war. The nitrogen mustard now widely used in therapeutics is bis (2-chlorethyl) methylamine hydrochloride (HN_2).

Mode of action: Nitrogen mustard (HN_2) reacts chemically with the DNA molecule and is especially active against proliferating cells, both normal and neoplastic. Lymphoid tissue and bone marrow are particularly susceptible to its action, and tumors of cells of the lymphoid series are relatively responsive to HN_2 therapy. Because of the similarity of its action on growing cells to that of X-rays, it is classed as a 'radiomimetic' agent. HN_2 is stable as a dried powder, but once in solution forms the chemically reactive unstable ethylene immonium cation, which is capable of reacting with a variety of chemical radicals, replacing the hydrogen in the reacting chemical by an alkyl group. This chemical reaction is known as alkylation.

Dose and administration: HN_2 is administered intravenously. It is extremely irritant to extravascular tissues and hence is administered into the tubing or side arm of a fast-flowing intravenous infusion of saline. Because of the common occurrence of nausea, premedication should be given, and further antiemetic treatment may be necessary. Antiemetic regimens vary considerably, but 10 mg metoclopramide intravenously immediately before chemotherapy, followed by 10 mg orally every 6 hours, if often effective. Alternatively, lorazepam, haloperiodal, or chlorpromazine may be used. Most of the combinations of chemotherapy currently in use for lymphoma treatment require antiemetic treatment.

The toxicity of HN_2 is directly related to dosage. Acute gastrointestinal upset, including nausea, vomiting, anorexia, and sometimes diarrhea, may begin within a few minutes of injection, and is generally much less after 4–6 hours. With high dosages, however, anorexia may persist for several days. The severity of bone marrow depression, in terms of the degree of neutropenia and thrombocytopenia, is usually reached 10–14 days after a single large dose of HN_2, and recovery to normal counts occurs in most instances by about 4 weeks.

Cyclophosphamide

This substance has the same alkylating groups as nitrogen mustard, attached to a cyclic phosphorus compound, but becomes active only after enzymatic cleavage of the ring structure. Such enzyme activity is high in many malignant tumors and may produce a high local alkylating effect at the tumor site compared with other tissues. However, enzyme activity is also present in liver and other normal tissues. Cyclophosphamide can be administered orally, directly into serous cavities, or injected into veins without special precautions.

Dosage and administration: The drug is supplied in ampoules as it is stable in solution, or in coated tablets of 50 mg. It is often injected intravenously in doses of 750–1500 mg/m^2, and causes less nausea in comparison with HN_2. Leukopenia regularly occurs, and generally reaches a crest approximately ten days after such administration. Erythropoiesis is also suppressed, but platelets generally decrease less with this drug than with other alkylating agents. Alopecia is very common and may be total. With either regular or very high-dose administration there is a tendency to develop hemorrhagic cystitis. This complication is less common if the drug is administered early in the day and the patient is given a large fluid intake together with a diuretic such as frusemide to flush the toxic metabolites from the bladder.

Oral administration of the drug on a long-term basis is generally in a dose of 50–100 mg/m^2, but the dose is titred according to the marrow tolerance of the individual patient. With such treatment, the onset of hemorrhagic cystitis may be gradual, and may be foreshadowed by the development of nocturia, reflecting fibrosis of the bladder wall and submucosal tissues.

Chlorambucil

This alkylating agent is restricted to oral usage. It is usually given in a regular daily dose of 0.05–0.2 mg/kg bodyweight, depending on hemopoietic tissue tolerance. Chlorambucil seldom causes gastrointestinal upset. Its principal side-effect is bone marrow depression, which is less readily reversible than in the case of cyclophosphamide or HN_2. Anemia, leukopenia, and thrombocytopenia, when they occur, tend to develop slowly with continuous daily therapy, and after the drug is withdrawn, only slow improvement in peripheral blood count can be expected over periods of up to several months.

Chlorambucil is most commonly administered as a single agent, or in combination with corticosteroids, for treatment of relatively well-differentiated lymphoma where suppression of disease activity results in substantial clinical benefit. Because of the prolonged nature of the marrow depressant effects it is not commonly used in combination chemotherapy.

Melphalan

This drug is administered by the oral route, and is associated with only minor gastrointestinal side effects except when used in high dosage, when such symptoms may be troublesome in occasional patients. It is available in 2 mg and 5 mg tablets, and

a convenient method of administration is intermittent courses at relatively high dosages of 9 mg/m^2/day for 4 days, every 4–6 weeks. The ensuing neutropenia reaches a peak in 2–3 weeks, and should recover before another course is administered at the same dosage. Melphalan may also be given on a daily basis in a dosage of 0.05–0.1 mg/kg.

The drug has few serious side effects, apart from the reversible bone marrow depression noted above, and a definite association with secondary leukemia. It is often effective in plasmacytoid lymphocytic neoplasia, and is used in the treatment of multiple myeloma.

Nitrosoureas

The nitrosoureas –1,3-bis (2-chlorethyl)-1-nitrosourea (BCNU) and 1-(2-chlorethyl)-3-cyclohexyl-1-nitrosourea (CCNU)- have been incorporated into combination chemotherapy as they have a spectrum of activity similar to nitrogen mustard and cyclophosphamide. They function as bifunctional alkylating agents. Two specific features are their ability to cross the blood-brain barrier, and their tendency to cause delayed and rather prolonged myelosuppression. Following a single dose, the peak of neutropenia and thrombocytopenia may be up to 6 weeks. BCNU is administered by intravenous infusion in doses of 100–200 mg/m^2 as a single agent, and is about half this dose when administered in combination with other cytotoxic drugs. CCNU is administered as a single oral dose of 130 mg/m^2, the dose also being reduced when the drug is used in combination with other cytotoxic agents.

Vinca Alkaloids

The vinca alkaloids, vincristine and vinblastine, are two of a variety of antineoplastic alkaloids derived from the periwinkle flower, Vinca rosea. They are very similar in their action, and one of the toxic effects is disruption of the mitotic spindle, causing metaphase arrest in dividing cells.

Vincristine is readily soluble in aqueous solution, and is stable as a freeze-dried powder. The usual dosage is up to 1.4 mg/m^2 for adults, and 2 mg/m^2 for children. It is available in 2 mg ampoules ready for reconstitution and may be given by direct intravenous injection, although great care should be taken as there is considerable irritant action if extravasation occurs. The compound is rapidly cleared from the circulation with a half-life of only a few minutes. Toxicity to the bone marrow is relatively minor compared with other cytotoxic drugs, and the major limiting factor is neurological toxicity. Alopecia is also a troublesome complication. Loss of deep tendon reflexes develops most prominently in the legs, and is usually seen only after three or four weekly doses of 2 mg in adults of normal size. However, occasionally unpleasant paraethesiae and signs of neuropathy develop even after the first dose, and such side effects appear to be more common in older patients. Abdominal pain and constipation may also occur as manifestations of automatic neuropathy, and disturbance of bladder function is also seen in a small proportion of patients. Variable and often complete recovery of neurological function occurs after cessation of treatment.

Vinblastine is a similar drug which is administered at about five times the dosage of vincristine. It is equally against lymphoid tumors, but at the dosage employed causes a greater degree of bone marrow suppression than vincristine. Neurological disturbance is less common with vinblastine than with vincristine, but the drug is slightly more irritant than vincristine and more inclined to induce nausea shortly after injection. Like vincristine, it is available as a freeze-dried powder, and ampoules contain 10 mg. When used as a single agent, it is ordinarily administered in a dose of 0.1–0.15 mg/kg, or 3.7 mg/m^2.

Both of these alkaloids are widely used in combination chemotherapy because of the relatively slight bone marrow toxicity they cause. Dosages and schedules for use, therefore, are governed by the drugs with which they are combined. It is advisable to use them not more than once every 7–10 days so that neurological toxicity may be evaluated before a further injection is given.

Epipodophyllotoxins

VP-16 (etoposide) and VM-26 (teniposide) are semi synthetic derivatives of podophyllotoxin, itself a mitotic inhibitor with unacceptable gastrointestinal toxic effects. VP-16 appears to arrest cells in late S or G2 phases of the cell cycle, while VM-26 prevents cells from entering mitosis. The drugs are administered by intravenous infusion, usually diluted in normal saline. Marrow suppression is the principal toxic effect, but hypotension may be associated with rapid administration. Fever and nausea are mild side effects. VM-26 can replace vincristine in combination chemotherapy regimens for treatment of lymphoma with comparable efficacy and reduced neurological side effects.

Procarbazine

Procarbazine is a derivative of methylhydrazine and acts in a manner that closely resembles the alkylating agents. It is effective when given by mouth, and is employed most commonly in a dosage of 50–150 mg/m^2/day (1–2.5 mg/kg) for periods of two or more weeks at a time. In addition to bone marrow suppression, which appears to be fully reversible on withdrawal of the drug, it produces central nervous system symptoms. Drowsiness, depression, nausea, and vomiting are not uncommon, particularly in the elderly. As the drug is a monoamine oxidase inhibitor, care must be taken to avoid foods such as cheese and chocolate. The drug also interferes with metabolism of alcohol, and patients should abstain from alcohol whilst on treatment.

A related drug is imidazole carboxamide, also known as dacarbazine (DTIC), a triazene alkylating agent. DTIC is administered intravenously and is employed in the 'ABVD' regimen for Hodgkin's disease. DTIC causes severe nausea and vomiting, as well as significant myelosuppression.

Other Drugs

Other drugs commonly employed in the chemotherapy of lymphomas are adriamycin (doxorubicin), cytosine arabinoside, methotrexate and corticosteroids. Interferon has produced some occasional beneficial responses and is undergoing further evaluation.

COMBINATION CHEMOTHERAPY FOR HODGKIN'S DISEASE

The usual form of combination therapy is the MOPP regimen. Usually six cycles of MOPP therapy are administered, and there is no evidence that further benefit is obtained from administration of further cycles. This particular protocol produced complete remission in 81% of the previously untreated patients with advanced disease considered unsuitable for radiation treatment. Subsequent experience has confirmed that this regimen can produce very long, complete remissions consistent with cure in at least 50% of patients with advanced stage Hodgkin's disease, nearly all of whom would have died in the past when available therapy was limited to radiotherapy or single chemotherapeutic agent administration. The MOPP regimen has become the benchmark against which other measures for treatment of Hodgkin's disease are compared (Table 14.4).

Table 14.4: MOPP combination chemotherapy	
Drugs	*Dosage*
Nitrogen mustard	6 mg/m^2 intravenously daily on days 1 and 8
Vincristine	1.4 mg/m^2 intravenously daily on days 1 and 8
Procarbazine	100 mg/m^2 orally daily from days 1 to 14 inclusive
Prednisone (with 1st and 4th courses)	40 mg/m^2 orally from days 1 to 14 inclusive

Six cycles are given with two weeks rest between completion of one cycle and commencement of the next. Modification of drug dosage may be necessary because of leukopenia or thrombocytopenia, as described in the original reference.

Myelosuppression is very commonly encountered during treatment with the MOPP regimen, and attenuation of the dosage of NH_2 and procarbazine is often necessary, especially in the latter cycles of treatment. Another very common side effect is induction of sterility. Males are almost always rendered sterile by a full course of treatment, and sterility develops in some females, usually in the older of the susceptible subjects.

Alternative chemotherapy protocols which produce similar results include ABVD (Adriamycin, bleomycin, vinblastine, and DTIC), and variations to the MOPP protocol, such as addition of bleomycin or replacement of nitrogen mustard with chlorambucil. Alternation of courses of MOPP and ABVD, or alternation of chemotherapy and radiation therapy appear to be promising approaches, but definitive studies are still required to establish whether any represent a significant advance over MOPP therapy alone.

There is, however, good evidence that therapy such as the ABVD regimen can produce sustained remissions with apparent cure in Hodgkin's disease which is unresponsive to MOPP therapy. ABVD treatment is also recommended for advanced stage disease which undergoes early relapse after MOPP therapy, but there is no evidence that ABVD is superior for disease which undergoes relapse after more than 12 months.

CHEMOTHERAPY FOR NON-HODGKIN'S LYMPHOMA

The various management options in non-Hodgkin's lymphoma are less clearly established than in Hodgkin's disease. Differing responses to treatment of the various histological subtypes have led to approaches that vary according to the histology, clinical state, age, and general condition of the patient. The sites of involvement with tumor and the size of tumor masses also influence the approach to treatment.

Some generalizations can be made. The favorable prognosis non-Hodgkin's lymphomas (the histological subtypes of nodular lymphocytic or nodular mixed cell, and diffuse well-differentiated), while having a generally benign natural history and good response to therapy, are curable only in the minority of cases. There is no logic in employing aggressive chemotherapy or radiotherapy of the type currently available, with their significant morbidity and even mortality, if the quality of life and survival of the patient is not influenced beneficially. This has led to widespread use of the following approach. The rather uncommon stages I and II in good-prognosis non-Hodgkin's lymphomas are treated with local intensive radiotherapy. More extensive stage III and IV disease is treated with chemotherapy aimed at suppression of disease activity and allevation of symptoms.

Sometimes, disease that shows no apparent progression in more elderly patients is kept under observation, and treatment is not instituted unless acceleration of disease activity takes place. Progressive or symptomatic disease is most commonly treated by cycles of combination chemotherapy such as the CVP regimen or by continuous oral administration of chlorambucil until a maximum response is obtained. It has not been established that

maintenance treatment provides any additional benefit after a complete remission has been carefully documented by staging procedures (Table 14.5).

The approach to the treatment of poor-prognosis histological subtypes is quite different

They usually progress rapidly, and advanced-stage unresponsive disease is frequently fatal within 6 months of diagnosis. Rapid cell turnover renders these tumors susceptible to 'cycle-specific' cytotoxic drugs and to radiotherapy.

Diffuse, poorly differentiated lymphocytic, diffuse mixed cell, and the 'histiocytic' or a large proportion, particularly of the diffuse large cell variety, can be cured. The best results in stages II, III and IV disease are with combination chemotherapy. The CHOP large cell lymphomas fall into this category. Stage I disease responds well to high-dose radiotherapy regimen has been widely employed with overall complete response rates of about 65% and an overall median survival times of 2 years. More aggressive regimens have been developed and are being evaluated. Inclusion of bleomycin, methotrexate, and cytarabine, and alternating the administration of drugs, may increase the efficacy of those already contained in the CHOP protocol (Table 14.6).

Table 14.5: CVP therapy for non-Hodgkin's lymphoma

Drug	*Dosage*	*Timing*
Cyclophosphamide	400 mg/m^2 orally*	Days 1–5
Vincristine	1.4 mg/m^2 intravenously	Day 1
Prednisone	100 mg orally	Days 1–5

Cycles of treatment are given every 3 weeks in the dosages stated. Neutropenia reaches a peak on days 7–14. The dosage of cyclophosphamide is modified, if necessary, according to white cell and platelet counts at the commencement of the next cycle.

*The 5 days of oral cyclophosphamide can be replaced by a single intravenous injection of 750 mg/m^2 cyclophosphamide on the first day of each cycle.

Table 14.6: CHOP regimen for poor prognosis non-Hodgkin's lymphoma

Drug	*Dose*	*Day*
Cyclophosphamide	750 mg/m^2 IV	1
Adriamycin	50 mg/m^2 IV	1
Vincristine	1.4 mg/m^2 IV	1
Prednisolone	100 mg orally	1–5

The regimen is repeated every three weeks, and these dosages may require modification according to bone marrow toxicity.

A specific subcategory of diffuse, poor-prognosis lymphoma is *T-cell lymphoblastic lymphoma.* This disease mostly affects young males, is T-cell derived, and presents with a mediastinal mass. Very aggressive treatment with regimens akin to acute lymphoblastic leukemia protocols is recommended for the therapy of this disorder.

Autologous bone marrow transplantation is currently undergoing evaluation as a means for enabling more intensive myelosuppressive chemotherapy to be administered to patients with lymphoma. Bone marrow from the patient is cryopreserved and then returned by intravenous infusion after normally lethal dosages of chemotherapeutic agents have been administered. The reinfused marrow restores hemopoietic tissue over the ensuing few months, and remissions have been obtained by this approach in lymphoma unresponsive to conventional dosages of chemotherapy.

OUTCOME IN MALIGNANT LYMPHOMA

The tremendously improved prognosis that follows radical radiation or chemotherapeutic management in responsive instances of this group of diseases has been described. Prognosis in Hodgkin's disease depends to an extent on the stage of disease when it presents. Patients with stage IA and IIA disease established by staging laparotomy, and who subsequently receive a full course of radiotherapy, have a survival at 5 years in excess of 85%, and although some relapses may occur for up to 10 years, the majority of these patients may be regarded as cured. Poor responses are commoner with lymphocyte depleted but no essential difference has been noted in remission rates between the other histological subtypes of the disorder. Adverse prognostic factors are disease in relapse after prior chemotherapy, the presence of a large mediastinal mass, advanced stage, and the presence of B symptoms.

Patients presenting with extensive Hodgkin's disease not amenable to radiation therapy, but treated with intensive combination chemotherapy, have complete remission rates of 75–85% after 6 months of MOPP treatment. Persistence of remission with values for reported, but most centres obtain values for disease-free survival of 50–60% in patients with extensive disease.

Non-Hodgkin's lymphoma has a much more variable outcome, and this is related to histological type.

Nodular and diffuse well-differentiated lymphocytic lymphomas have a relatively indolent course even in the untreated state, and in nodular lymphoma amenable to radiation treatment, median survival uncorrected for age is reported to be approximately 7.5 years.

Stage I and IE diffuse large-cell lymphoma treated by intensive radiotherapy also has a comparatively good prognosis, and approximately 75% of cases may be cured. More extensive disease may be cured by chemotherapy, but the overall prognosis is worse, the median survival ranging from 23 to 50 months with current regimens. More extensive and bulky disease, and increasing age are adverse prognostic factors, with stage IV large-cell lymphoma having a median survival of less than 12 months.

CHAPTER

15

Myeloproliferative Disorders

Myeloproliferative disorders are a group of conditions characterized by endogenous proliferation of one or more hemopoietic components in the bone marrow and in many cases by myeloid metaplasia (extramedullary hemopoiesis) in liver and spleen. These disorders are closely related, transitional forms occur and, in many patients, an evolution from one entity into another occurs during the course of illness) (Fig. 15.1). Polycythemia vera, essential thrombocythemia and myelosclerosis are collectively known as non-leukemic myeloproliferative disorders and are discussed here: The myeloid leukemias are discussed elsewhere.

POLYCYTHEMIA

Polycythemia (Erythrocytosis) refers to:

- Increase in hemoglobin
  - Above 17.5 g% in males
  - Above 15.5 g% in females
- Increase in red cell count
  - Above 6 million/mm^3 in males
  - Above 5.5 million/mm^3 in females
- Increase in hematocrit
  - Above 55% in males
  - Above 47% in females
- Increase in total volume of red cells
  - Above 25–35 mL/kg in males
  - Above 20–30 mL/kg in females

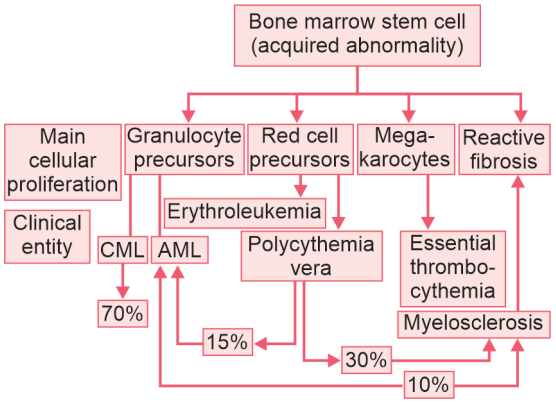


Fig. 15.1: Myeloproliferative diseases and their interrelationships

VARIOUS CAUSES OF POLYCYTHEMIA

Primary

Polycythemia vera.

Secondary

Associated with Hypoxia

a. Cardiovascular disease, usually congenital resulting in significant venous admixture
b. Pulmonary disease resulting in:
   - Impaired gas perfusion
   - Perfusion of poorly aerated lung
   - Pulmonary arteriovenous fistulas

c. High altitude residence
d. Hypoventilation associated with obesity (Pickwickian syndrome)
e. Hemoglobin variant with increased affinity for oxygen
f. Heavy smoking
g. Methemoglobinemia (rarely).

Due to Inappropriate Erythropoietin Increase in

a. Benign and malignant tumours of:
   - Kidney
   - Liver
   - CNS
   - Uterus
   - Ovary
b. Renal disease (besides malignancies)
   - Hydronephrosis
   - Vascular impairment
   - Cysts

Associated with Adrenocortical Steroids or Androgens

a. Adrenal hypercorticism (all types)
b. Virilizing tumors
c. Androgens used therapeutically (rarely corticoids)

Associated with Chronic Chemical Exposure

a. Nitrites, sulfonamides, other substances producing methemoglobin and sulfhemoglobin.
b. Cobalt, shellac components, various alcohols.

Benign Familial Polycythemia Relative

a. 'Stress' or 'spurious' polycythemia
b. Dehydration: Water deprivation, vomiting
c. Plasma loss; burns, enteropathy.

POLYCYTHEMIA VERA

Erythraemia, Vaquez–Osler disease, and polycythemia rubra vera are synonyms for polycythemia vera, which is a chronic, progressive, and ultimately fatal disease, in which the fundamental abnormality is an excess production of the formed elements of the blood by a hyperplastic bone marrow. The marrow hyperplasia is not secondary to any recognized bone marrow stimulus, and studies using isoenzymes for glucose-6-phosphate dehydrogenase indicate that the affected cells are members of a clone that has arisen from a single progenitor. Progenitors of red cells in polycythemia vera are unusually responsive to erythopoietin and there is an apparent appropriate reduction in the plasma concentration of erythropoietin in response to the elevated PCV. Polycythemia vera may be regarded as a relatively benign type of neoplasm of hemopoietic tissue, in which the dominant clinical manifestations are due to the abnormal increase in red cell precursor activity. The occasional occurrence of cytogenetic abnormalities in bone marrow cells, particularly trisomy 9, emphasizes its neoplastic nature. Similar features exist in myelofibrosis and essential thrombocythemia, emphasizing the relationship between these disorders. Polycythemia vera is classified as one of the myeloproliferative disorders and commonly evolves into myelofibrosis.

PATHOPHYSIOLOGY

The basic abnormality is hyperplasia of the precursors of the red cells, granulocytes, and platelets in the bone marrow, with resultant excess production of these cells. The overproduction of red cells results in an increase in the total red cell volume, sometimes to twice its normal value, or even more. This results in an increase in the number of red cells per litre of blood, and thus evaluated PCV, and in an absolute increase in the total blood volume of the body. The increased blood volume is due predominantly to the increase in total red cell volume, the plasma volume generally being within the normal range, although it is sometimes increased. The increased blood volume is accommodated mainly by capillary dilatation; at postmortem all the organs of the body are engorged with blood.

Excessive production of red cells is responsible for most of the symptoms of polycythemia vera, and, together with the excess number of platelets, for the vascular insufficiency which causes much of the morbidity and mortality. The increased blood volume causes a diversity of symptoms, the most prominent being cerebral. The increase in PCV, and the associated increase in viscosity of the blood, tends to slow the rate of blood flow, and predisposes to the thrombosis that so commonly occurs. Increased platelet adhesiveness may also contribute.

A hemorrhagic tendency also occurs. The cause is incompletely understood, but it is probable that several factors contribute, namely vascular engorgement and defects of platelet function similar to those of essential thrombocythemia.

CLINICAL PRESENTATION

Polycythemia vera is basically a disease of middle and old age, the majority of cases occurring between 40 and 80 years, with onset most frequently at about 60 years. It occurs occasionally in younger adults, and rare cases in the second decade have been described. Males are affected a little more commonly than females.

The clinical picture (Table 15.1) is influenced by the severity and rate of progress of the disorder, and by the number and type of complications. Symptoms are caused mainly by the increased blood volume and by the thrombotic and hemorrhagic complications. The increased blood volume causes engorgement and slowing of the circulation in many organs; therefore, symptoms may be referred to a number of systems.

Table 15.1: Presenting features of polycythemia vera

Common
• Cerebral symptoms, especially headache and dizziness
• Cardiovascular symptoms
• Development of red face or bloodshot eyes
• Weakness, lassitude, and tiredness
• Gastrointestinal symptoms, especially dyspepsia
• Visual disturbances
• Pruritus
• Thrombotic complications
• Hemorrhagic manifestations
• Peripheral vascular disease
• Accidental discovery on routine examination
Occasional
• Splenomegaly
• Gout
• Incidental discovery in investigation of other medical problems
• Psychiatric manifestations

The onset is usually insidious, often with vague symptoms referred to one or more of the systems mentioned below. The most common presentation is with cerebral symptoms. Occasionally, an acute thrombotic or hemorrhagic complication causing a medical or surgical emergency is the presenting manifestation. When asymptomatic, the disorder is sometimes accidentally discovered on routine physical examination.

Central Nervous System

Cerebral symptoms occur in most patients, and are the most common presenting manifestation. Headache, fullness in the head, and dizziness are the usual complaints, but visual symptoms, tinnitus, syncope, loss of memory, inability to concentrate, and irritability also occur. Headache may be mild or severe, frequent or occasional, and varies in location from frontal to occipital. It may be worse on awakening in the morning or on lying down. Depression and other psychiatric disturbances occur occasionally.

Cerebrovascular accidents vary from mild attacks causing transient weakness of a limb, loss of consciousness or dysphasia, to the classical picture of a major cerebral thrombosis or hemorrhage. They are a common cause of death.

Weakness, lassitude, fatigue, and weight loss are common symptoms.

Cardiovascular System

Cardiac symptoms are frequent, dyspnea being the most common. Because of the age group in which polycythemia occurs, degenerative arterial disease and essential hypertension are frequent associations, and probably contribute largely to the cardiovascular manifestations. Hypertension is present in about 50% of cases, but because it is corrected in only a few cases after adequate therapy, it is probably due to essential hypertension, the increased blood volume making relatively little contribution. In the normotensive patient, the heart is usually of normal size. Angina of effort, coronary insufficiency, and cardiac failure are important and common complications.

Peripheral Vascular Disorders

Peripheral vascular disorders of varying types are frequent. The result from slowing of the circulation, thrombosis, and associated atherosclerosis. Erythromelalgia, arterial thrombosis, thromboangitis obliterans, superficial and deep venous thrombosis, and Raynaud's phenomenon may occur. Pain in the extremies, including intermittent claudication, may be prominent symptom. Arterial occlusion may result in gangrene.

Gastrointestinal Symptoms

Especially dyspepsia and flatulence, occur frequently. Symptoms are due mainly to the vascular engorgement of the alimentary tract, but about 10% of cases have a radiologically or endoscopically demonstrable peptic ulcer, usually duodenal. Abdominal pain may result from peptic ulceration, splenic enlargement, or infarction, or occasionally from mesenteric thrombosis. Hemorrhage from the congested mucosa of the stomach and bowel, or from a peptic ulcer, is not uncommon. Occasionally, the patient actually presents with anemia resulting from occult gastrointestinal bleeding. Mild weight loss is common.

Visual Disturbances

Visual disturbances are common; they result from engorgement of retinal veins and sometimes from thrombosis or hemorrhage. Scotomata, spots before the eyes, and transient dimness of vision are most common, but temporary blindness or diplopia may occur.

Thrombosis and Hemorrhage

Particularly thrombosis, are important causes of both morbidity and mortality. Thrombotic manifestations include cerebral and coronary thrombosis, mesenteric thrombosis, thrombosis of peripheral arteries, and pulmonary thrombosis. Postoperative thrombosis is common. More than 75% of patients with uncontrolled polycythemia develop bleeding or thrombotic complications following major surgery, and elective procedures should never be undertaken prior to control of the polycythemia. Thrombosis and hemorrhage occur more frequently in patients over the age of 70 years.

Gout

It occurs in about 10% of cases, and elevated plasma urate in more than 50% the gout may be temporarily exacerbated by treatment. The first attack of gout may precede the diagnosis of polycythemia vera by some years. Uric acid nephropathy with diffuse deposition of uric acid crystals through the kidney may occur, and in some cases there is calculcus formation. Bone pain also occurs occasionally.

Generalized Pruritus

Often worse on the palms and soles, and aggravated by hot baths, occurs in about half the cases; it is an important symptom in diagnosis, especially in differential diagnosis from secondary polycythemia in which it rarely, if ever, occurs. The pruritus is considered to be due to liberation of histamine from the basophil granulocytes. Paresthesiae with numbness and tingling may also occur.

GENERAL PHYSICAL EXAMINATION AND X-RAY

On examination, the outstanding features are the red color of the skin and mucous membranes, congestion of the conjunctival vessels, and engorgement of the retinal veins. Splenomegaly is present at the time of diagnosis in some 70% of cases.

Skin and Mucous Membranes

The red color of the skin and mucous membranes is a striking feature in most, but not all, patients. The skin in florid cases is typically a brick-red color, often with a dusky cyanotic hue which is more marked in cold weather. The high color results from the marked engorgement and distension of the superficial capillaries. Easily recognized telangietasia on the cheeks is common. The nail beds and palms of the hands are useful sites for assessment of the degree of plethora. The skin is warm, and the superficial veins are often distended. The mucous membranes of the mouth and tongue are often a deep red color.

Conjunctival Vessels

The conjunctival vessels are usually injected. Excess lacrimation may occur, but pain or soreness of the eyes is rare. It is not uncommon for the patient to present complaining of bloodshot eyes, or to give a history of having been treated for conjunctivitis. Ophthalmoscopic examination reveals a deeply colored retina with engorged, tortuous veins. Retinal thrombosis and hemorrhage are sometimes seen.

Splenomegaly

Splenomegaly is present in 70% of cases initially, and may subsequently develop in others. It is usually only of mild to moderate degree, although it is occasionally marked. The

spleen is smooth and firm, and relatively rapid enlargement suggests the development of myelofibrosis or leukemia. Infarction of the spleen may cause perisplenitis with pain and sometimes a friction rub. Hepatomegaly, either slight or moderate, is often present.

Chest X-ray

The chest X-ray may show prominent pulmonary vessels. The large number of visible end on vessels may produce mottling, particularly in the lower and mid-zones of the lungs, well out to the periphery. Previous pulmonary infarction may have resolved completely, or may have left areas of plate atelectasis or linear scars.

Peripheral Blood Picture

Blood obtained by venepuncture is dark, thick, viscous, and clots readily. The increased viscosity may make the spreading of satisfactory films difficult.

Red Cell Count

The red cell count is raised, usual values being 8–10 × 10^{12}/L, and with counts up to 12 × 10^{12}/L occurring occasionally. The *hemoglobin* level is usually in the range of 18–24 g/dL, although it may be higher. The MCH is often slightly reduced, and thus the relative increase in hemoglobin level may be little less than the degree of increase in red cell count. The PCV is raised, usually 0.60–0.70, and sometimes higher. The MCV is usually in the lower normal range, but may be reduced, especially when there has been gastrointestinal bleeding.

In the film of an uncomplicated cases, the red cells usually appear normal. There is sometimes slight anisocytosis and microcytosis. A few round polychromatic macrocytes and an occasional nucleated red cell may be seen. Reticulocytes are 1.5% or above in half the cases. In patients who have had repeated venesections or spontaneous bleeding, features of iron deficiency may be prominent.

Leukocytosis

Leukocytosis is present in nearly half the cases at presentation, the white count usually being from 12 to 20 × 10^9/L, though occasionally counts up to 50 × 10^9/L occur. There is usually a shift to the left with metamyelocytes and stab forms, and often occasional myelocytes.

Neutrophil Alkaline Phosphatase

The neutrophil alkaline phosphatase is increased in more than 70% of cases, but correlates poorly with the white cell count.

Platelet Count

The platelet count is above 400 × 10^9/L in nearly two-thirds of cases, most frequently ranging from 400–800 × 10^9/L, but occasionally reaching several 1000 × 10^9/L. Macrothrombocytes may be seen. The blood clotting times is not prolonged, but the clot is bulky and may be fragile. The bleeding time is usually normal, but is occasionally prolonged.

Erythrocyte Sedimentation Rate

The erythrocyte sedimentation rate is very often low, usually being not more than 1 mm/hour.

Biochemical Parameters

The serum bilirubin value is commonly at the upper limit of normal, but is sometimes slightly raised. Plasma iron and ferritin levels may be decreased, and are especially so after venesection. The serum level of vitamin B_{12}, unsaturated B_{12}-binding capacity, and lysozyme are commonly elevated. reduction in serum folate levels may occur occasionally, a reflection of mild deficiency secondary to erythroid hyperplasia. Blood histamine values are often raised, and the activity of the enzyme histidine decarboxylase, which is responsible for histamine synthesis, is increased in leukocyte-rich blood fractions.

The plasma protein levels, including the fibrinogen concentration, are usually normal. The serum uric acid is raised in over half the cases. The serum lactic dehydrogenase activity is usually normal, as is the serum haptoglobin level.

When the disorder evolves into myelofibrosis, the peripheral blood picture of that condition develops. The hemoglobin level progressively falls, moderate to marked anisocytosis and poikilocytosis appear, and the number of myelocytes and normoblasts increases, together with the degree of polychromasia. There may be an elevated total white cell count, with a shift to the left, but at times the white counts fall and leucopenia develops. The platelet count sometimes remains high, but often falls to normal, or less than normal, as the disease evolves.

Bone Marrow Examination (Fig. 15.2)

Hyperplastic bone marrow is evident on trephine biopsy. There is replacement of fat cells in the usual sites of hemopoiesis, giving the marrow a darker red appearance than normal, and an extension of the red marrow down the shafts of the long bones which normally contain yellow marrow. Trephine specimens commonly show some increase in reticulin.

Aspirated marrow usually contains numerous fragments, which low-power examination shows to be densely cellular and to contain either no fat or much less fat than normal. The cell trails are usually hypercellular. Erythropoiesis is normoblastic, and numerous clumps of developing normoblasts are prominent. Granulopoiesis is active, and since it shares in the hyperplasia, the myeloid:, erythroid ratio is usually within normal limits, although it is sometimes reduced. Megakaryocytes are increased in number, and because of their size, are a prominent features of the marrow. They often occur in clumps of 2–5 or even more, and are most obvious in the region of marrow fragments and at the margins of the film. Many megakaryocytes have platelets masses attached. Sometimes, aspiration of particles is difficult or impossible, and this is not uncommonly related to more extensive development of reticulin, or extreme hypercellularity of the marrow.

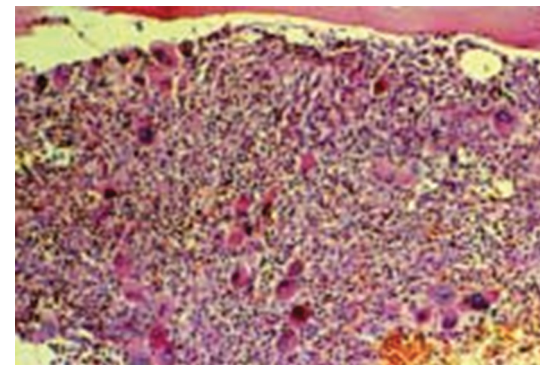


Fig. 15.2: Polycythemia vera, bone marrow core biopsy

ESTABLISHING DIAGNOSIS

In well-developed cases, the diagnosis is usually obvious from the presence of the classical triad—a dusky brick-red color of the face ('ruddy cyanosis'), splenomegaly, and an elevated hemoglobin level with leukocytosis and thrombocytosis. However, it must be remembered that splenomegaly, lecukocytosis and thrombocytosis are absent in a proportion of cases.

Pruritus for which there is no other obvious cause in a polycythemic patient strongly suggests polycythemia vera. Similarly, a raised neutrophil alkaline phosphatase in a polycythemic patient also strongly suggests polycythemia vera, provided that infection and other causes of increased alkaline phosphatase are absent.

Total red cell mass should be measured in most cases in order to exclude relative polycythemia.

Diagnostic problem may occur in those cases in which the red cell values are only slightly increased, or are in the upper normal range. This may occur in the early stages, or when the polycythemia is 'masked' by a complicating factor, such as consequences of occult intestinal bleeding. An increase in plasma occurs typically in cases complicated by congestive cardiac failure, but may occur without cardiac failure.

Rare cases actually present with anemia due to gastrointestinal bleeding. The anemia may be either normochromic, or hypochromic due to iron deficiency. Such a situation must be differentiated from anemia due to the development of myelofibrosis.

Polycythemia Study Group Criteria for Diagnosis of Polycythemia Vera

Category A (Major Criteria)

1. Elevated red cell mass
2. Normal arterial oxygen saturation
3. Splenomegaly

Category B (Minor Criteria)

1. Leukocytosis
2. Thrombocytosis
3. Elevated leukocyte/neutrophil alkaline phosphatase score
4. Increased serum vitamin B_{12} or vitamin B_{12} binding proteins.

To establish a diagnosis of polycythemia vera, either all three diagnosis criteria from category A or an elevated red cell mass and normal arterial oxygen saturation in addition to two criteria from category B must be present.

Differential Diagnosis (Table 15.2)

Differentiation must be made from other disorders with similar clinical manifestations, and from other causes of a raised hemoglobin level.

In cases with an insidious onset, the vague and often somewhat indefinite symptoms such as headache, dizziness, fullness of head, weakness, and lassitude may not suggest a specific diagnosis. On the other hand, when symptoms point mainly to one system or organ, diagnosis of primary disease of that system or organ may be made and the underlying polycythemia overlooked. Thus, a primary diagnosis of congestive cardiac failure, essential hypertension, coronary artery disease, peptic ulcer, functional dyspepsia, peripheral vascular disease, phleobothrombosis or thrombophlebitis, cerebrovascular accident, mesenteric infarction, conjunctivitis, or gout may be made.

Pseudopolycythemia

Pseudopolycythemia may be confused with polycythemia vera, especially early and 'masked' cases, in which the red cell count is not markedly raised. The plethoric facies of the two conditions may be indistinguishable. Pseudopolycythemia lacks certain clinical features of polycythemia vera—the typical ruddy color of the mucous membranes, marked engorgement of retinal veins, splenomegaly, pruritus, leukocytosis, thrombocytosis, and

Table 15.2: Differential diagnosis—features of polycythemia vera, secondary polycythemia, and pseudopolycythemia

	Polycythemia	*Hypoxic secondary*	*Secondary erythrocytosis*	*Pseudopolycythemia*
Etiology	Neoplastic	Hypoxia due to underlying disorder—pulmonary and cardiac disease the most common causes	Inappropriately increased erythropoietin production-associated with renal and certain other tumors	Unknown. Anxiety state hypertension, obesity commonly associated
Clinical features				
Facies	Brick-red color in severe cases	Bluish cyanosis in more severe cases	Brick-red color in more severe cases	Brick-red color in more severe cases
Oral mucous membranes	Ruddy cyanosis	Bluish cyanosis	Normal or ruddy cyanosis	Normal
Conjunctival vessels	Injected	Injected in more severe cases	Injected in more sever cases	Injection absent or slight
Retinal vessels	Engorged	Engorged in severe cases	Engorged in severe cases	Not engorged
Spleen	Palpable (75% cases)	Usually impalpable	Usually impalpable	Usually impalpable
Pruritus	Common	Absent	Absent	Absent
Blood examination				
Red cell count, hemoglobin, and PCV	Mild to marked increase	Increase usually mild to moderate	Increase usually mild to moderate	Increase usually mild
White cell count	Raised (75% cases)	Normal	Normal	Normal
Platelet count	Raised (66% cases)	Normal	Normal	Normal
ESR	1 mm/hour or less	About 1 mm/hour except in severe cases	About 1 mm/hour except in severe cases	About 1 mm/hour
Neutrophil alkaline phosphatase	Usually but not invariably increased	Normal (may be increased by infection)	Normal	Normal
Arterial oxygen saturation	Normal	Reduced	Normal	Normal
Blood volume studies				
Red cell volume	Increased	Increased	Increased	Normal
Plasma volume	Usually normal, but may be reduced or increased	Normal or slightly reduced	Normal or slightly reduced	reduced

raised neutrophil alkaline phospatase; however, as any or all of these may be absent in polycythemia vera, especially in the early stages, distinction can often be made with certainty only by red cell and plasma volume determinations. If blood volume determinations cannot be performed, careful clinical and hematological observations over a period of months or years may be necessary. In pseudopolycythemia, the blood parameters remain relatively static, while in polycythemia vera the hematological changes progress, and typical features appear in time. The usual error is for pseudopolycythemia to be diagnosed as polycythemia vera and treated as such.

Secondary Hypoxic Erythrocytosis

Secondary hypoxic erythrocytosis can usually be distinguished by the presence of clinical manifestations of an underlying cause of hypoxia (most often a pulmonary or cardiac lesion), by the normal white cell and platelet counts, and by the absence of splenomegaly. When doubt exists as to whether the spleen is enlarged, an isotopic liver/spleen scan is of value. The white cell count may be raised by a complicating infection, especially in pulmonary disease.

Difficulty arises where central cyanosis is not prominent at rest. It may become more so on exercise. This occurs most commonly with pulmonary disease such as emphysema and pulmonary fibrosis, particularly in obese patients. It should be remembered that in some cases of congenital heart disease associated with reversed shunt, and in some cases of pulmonary arteriovenous fistula, cyanosis and polycythemia do not develop until adult life.

Estimation of the arterial oxygen saturation is important in diagnosis; it is usually normal in polycythemia vera, and below 90% in secondary polycythemia. Once it is established that the polycythemia is of the secondary type, appropriate investigations can be carried out to determine the cause of the hypoxia if it is not obvious.

Renal Erythrocytosis

The possibility of a renal cause should be considered in all patients with erythrocytosis but no leukocytosis, thrombocytosis, splenomegaly, or hypoxia, it should especially be considered in the polycythemic patient with hematuria, although it must be recognized that hematuria can occur as a complication of polycythemia vera. Pruritus is absent in renal polycythemia, and the neutrophil alkaline phosphatase is normal unless a sizable renal carcinoma is present.

Erythrocytosis with cerebellar tumor. Polycythemia vera with headache and papilledema, or with headache and papilledema, or with cerebellar signs due to vascular accident, must be differentiated from the rare association of erythrocytosis with cerebellar hemangioblastoma. The tumor is uncommon, but 15–20% have some degree of elevated hemoglobin level.

COURSE AND PROGNOSIS

The natural history without treatment is of chronic, progressive, and ultimately fatal disorder. It can be divided into three phases:

1. The onset phase, before the red cell volume has been much increased, is relatively asymptomatic and usually lasts for some years. A careful history at the time of diagnosis commonly reveals that mild symptoms have been present for several years prior to diagnosis.
2. The erythremic phase, when the classical signs and blood picture develop, shows considerable individual variation in both severity of symptoms and rate of progress.

In the majority of untreated cases, symptoms and signs slowly progress, but the course may be punctuated by acute episodes due either to thrombosis or hemorrhage, which can be fatal. In a few cases, the clinical manifestations and hematological picture remain relatively stationary for some years. This second phase lasts from several years to ten years or more.

3. The spent or 'burnt-out' phase, which ultimately occurs in patients who survive the vascular complications of the second stage, is associated with the development of myelofibrosis which not uncommonly is complicated by leukemia.

Myelofibrosis is a common terminal event, but may develop early in some cases. There is frequently a leukoerythroblastic anemia with anisocytosis and poikilocytosis, and a progressive and usually marked enlargement of the spleen. Occasionally, the blood picture is that of pancytopenia. The onset is usually relatively slow, the patient having been in remission for some time following the last course of treatment. Marrow aspiration yields a 'dry' or 'blood' tap, and marrow trephine biopsy shows the typical picture of myelofibrosis.

Leukemia is estimated to develop in at least 10% of cases, and is considered to be largely the result of the use of radioactive phosphorus (32P) or alkylating agents. However, it also occurs, uncommonly, in patients treated by phlebotomy alone. It is usually of the acute meyloid variety, although the picture can resemble that of certain of the myelodysplastic disorders.

The major causes of death are thrombosis and hemorrhage (especially cerebral thrombosis and hemorrhage, coronary occlusion, and gastrointestinal hemorrhage), congestive cardiac failure, marrow failure, and leukemia. Occasionally death occurs from some unrelated disorder, e.g. carcinoma. Before the introduction of 32P treatment, the average duration of survival after diagnosis was 5–7 years, the usual causes of death being thrombosis and hemorrhage. With the use of 32P, or busulphan or other alkylating agents, and the consequent reduction of vascular complications, the average period of survival has lengthened to 10–15 years.

TREATMENT

It has been pointed out that the majority of symptoms and complicatons of polycythemia vera are due to two factors, namely the increased blood volume, and the tendency to thrombosis and hemorrhage, factors which are a direct result of the excess production of red cells and platelets by the hyperplastic marrow. Therefore, the principle of treatment is reduction in the red cell mass by venesection, followed by suppression of bone marrow cell production. When suppression of blood cell production has been achieved, symptoms are alleviated and the incidence of vascular complications falls from more than 40 to less than 5%.

Suppression of bone marrow activity can be achieved either by intravenous injections of radioactive phosphorus (32P), or by chemotherapy with myelosuppressive drugs. Venesection to reduce the red cell mass is the most urgent initial treatment in a severely polycythemic patient, as it can rapidly reduce blood viscosity before a response to any other definitive treatment can be achieved.

Plan of Therapy in the Individual Patient

On diagnosing polycythemia vera, three basic approaches to therapy are available: (a) venesection plus measures to depress marrow activity; (b) therapy to depress bone marrow activity; and (c) venesection alone.

The choice of treatment is based on two main factors: the degree of increase in red cell mass as judged by its effect on the hematocrit, plus the symptoms and signs that it produces; and the degree of thrombocytosis.

When the platelet count is raised and the patient has symptoms, marrow suppressive therapy should be used. When the PCV is increased above 0.55, or if symptoms are troublesome, preliminary venesection should be performed. The majority of patients at the time of diagnosis require repeated venesection followed by marrow suppressive therapy. In the occasional patient with mild erythrocytosis and a normal platelet count, venesection alone may be sufficient for a considerable period of time before active progression of the disease warrants the introduction of myelosuppressive therapy.

The same considerations apply when active progression of the disease resumes after a phase of effective myelosuppression. However, with careful follow-up, the increase in activity is detected early, and sometimes there is only a moderate increase in PCV and no thrombocytosis. In such cases, venesection alone may be sufficient treatment for months or even years.

It is important that myelosuppressive therapy be given as soon as a marked increase in platelet count occurs, as some studies suggest the incidence of thrombotic complications is greater in patients with thrombocytosis.

Choice of Marrow Depressive Agent

Most often used, radioactive phosphorus is the simplest form of myelosuppressive therapy. Its advantages include ease of administration, simple follow-up, and more certain prediction of the effects resulting, from a particular dose. The disadvantage is the higher incidence of leukemia in long-term survivors in comparison with patients who receive no agents with mutagenic potential. It remains the treatment of choice for patients aged over 60 years with a requirement for myelosuppressive treatment.

Chemotherapy

The cytotoxic agents most used have been chlorambucil, busulphan, and hydroxyurea. The initially postulated advantage of chemotherapy over ^{32}P was possibly lower incidence of leukemia, but it is now established that chlorambucil therapy is significantly associated with the development of leukemia. Busulphan therapy is probably associated with an incidence of leukemia approaching that seen with ^{32}P and it is too early to be certain whether hydroxyurea is similar in this respect. Chemotherapy has certain disadvantages: the patients must take tablets for weeks or months, and the response to a given dose is less predictable than with ^{32}P. Blood counts must be performed frequently, especially in the initial stages, so that dosage can be adjusted. The dosage of the drugs is usually slightly less than that used for treatment of chronic granulocytic leukemia.

To summarize, when myelosuppressive therapy is required, ^{32}P is the agent of choice in patients over the age of 60 years, while chemotherapy should be considered in younger patients. Chemotherapy is indicated in patients who develop resistance to ^{32}P.

Radioactive Phosphorus

^{32}P was introduced in 1938 to control the disease activity, and has proved an effective and generally nontoxic method of treatment. Following its administration, radiation of the marrow cells results from the uptake of ^{32}P into the nucleic acids of mitotically active marrow cells, and the incorporation of ^{32}P into bone.

Dose

^{32}P is administered intravenously in the form of an isotonic solution of sodium phosphate. The initial dose varies from 111 to 185 MBq (3–5 mCi), depending on the severity of the disorder and the size of the patient; the higher the counts and the heavier the patient, the greater the dose required.

Toxic effects

The injection is free from side effects or radiation sickness. Pancytopenia is a possible complication, but is rare with usual therapeutic doses. Platelet production is most sensitive to ^{32}P, and treatment is occasionally follwed by thrombocytopenia. After repeated courses, there is occasionally a gradual, persistent fall in platelets or white cells, in which case further ^{32}P should be withheld and the patient managed by vensection.

Leukemia as a terminal event is largely the result of radiation from ^{32}P. The incidence is dose related, i.e. the risk of developing acute leukemia increases with the total dose of ^{32}P. The incidence of acute leukemia in patients treated with radiation is up to 10%, contrasted with about 1% in patients treated by venesection. However, it must be emphasized that vascular complications are readily controlled with this treatment, and for older patients the ease of administration and low intensity of posttreatment supervision means that it remains an attractive choice, especially as earlier studies indicate the overall survival in this group is greater with ^{32}P treatment than with venesection alone.

Response and Follow-up

Response to therapy is assessed by regular clinical and hematological follow-up examinations. For practical purposes, it is sufficient to perform blood counts, 6, 12, and 16 weeks after the administration of ^{32}P. The rate of response of the three formed elements of the blood depends in part on their life span.

Fall in relatively short-lived platelets and white cells occurs well before that of the red cells, with their life span of 120 days. Platelet reduction usually occurs at the end of the third week; the count reaches its minimum in 4–6 weeks (usually about 100×10^9/L), after which a rise occurs to normal values. The white cell count usually falls along with the platelet count, and tends to rise more slowly. Significant fall in the PCV and red cell count is usually obvious by the sixth week, and is maximal in 3–4 months. An earlier fall occurs, of course, if venesection is also used.

After 3–4 months, the hematological and clinical responses are assessed. The vast majority of patients have a satisfactory response, with relief of symptoms, reduction in size of the spleen, which may become impalpable, and reversion of the blood picture to normal or near normal. Most symptoms, including headache, fullness in the head, dizziness, weakness, dyspnea on exertion, and pruritus, are usually relieved. Dyspepsia, when due to peptic ulcer, may persist, and persistent pruritus can be a problem. Hypertension, when present, is usually not eliminated. If a satisfactory resmission has not occurred, further treatment is given, and the response followed as before. Patients who have a satisfactory response should be seen at intervals of three months, and assessed for signs of recurrent disease activity. Blood examination should always include a platelet count. Remissions last from 6 months to more than 5 years, the average being about 18–24 months. It is quite common from the duration of remission in an individual patient to remain fairly constant after each successive treatment.

A further course of therapy is indicated when there is rise in platelet count above normal, or a rise in PCV that is not readily controlled by venesection. With an adequate

follow-up, relapse is detected early, and the further doses required, especially the control of thrombocytosis alone, are often relatively small.

Resistance to ^{32}P

In some patients, the response of the red cell count to the usual doses of ^{32}P is unsatisfactory; about 10% show only partial improvement, and 5% no improvement. In such patients, the larger doses of ^{32}P required to control red cell production predispose to thrombocytopenia, so that it is preferable to control the red cell mass by venesection and the thrombocytosis with appropriate doses of ^{32}P. Sometimes, chemotherapy may provide more satisfactory control of the disorder in this setting than ^{32}P.

Chemotherapy

The various cytotoxic agents which have been used in the treatment of polycythemia vera include *busulfan, chlorambucil, melphalan,* and hydroxyurea. A piperazine derivation pipobroman has also been successfully employed.

Chlorambucil has now been shown to give rise to a greater incidence of leukemia than ^{32}P and hence should not be used. The same is reported to be the case with melphalan. Busulfan has a better record in this respect is most published trials but it is too early to be certain of the degree of risk. Hydroxyurea has the advantage that it is not an alkylating agent, and preliminary trials show promise. Pipobroman is an effective drug, but it is too early to be certain of its oncogenic potential.

Polycythemia vera is more sensitive to busulfan than is chronic myelocytic leukemia. The usual maximum regular daily dose recommended for the latter disorder is 6 mg, and therefore the safest regimens for polycythemia and those of 4 mg or less per day. Thus a dose of 4 mg/day may be given for the period of 4–12 weeks, depending on the observed response, and after normal blood indices are obtained, the count are monitored and maintenance therapy should not be given. During treatment, the rate of fall of all three blood cell series should be carefully noted, and it is recommended that busulfan be stopped when the platelet count reaches 300×10^9/L, as the count may fall considerably after the drug is discontinued. Busulfan has a marked effect on production of platelets and is thus of value in patients with marked thrombocytosis. The marrow suppression produced by hydroxyurea is more rapidly reversible than that produced by busulfan, so that hydroxyurea is preferred in patients with normal or low platelet and white cell count. It is given initially in a dose of 1.5 g daily to an adult of normal size, and the dosage adjusted according to tolerance. As its effects are relatively transient, ongoing therapy is usually required and prolonged untreated remissions are less common than with busulphan. Pipobroman is used in a dose of 50 or 75 mg daily over 5–10 weeks.

Venesection

Venesection is the most direct and simple method of treatment. It is especially efficacious in producing rapid relief of symptoms caused by increased blood volume or viscosity. However, it does not reduce the platelet count or have any effect on the underlying disorder, and thus rarely proves adequate as the sole method of treatment throughout the entire course of disorder. The high incidence of vascular complications in patient treated by this means alone is not reduced by platelet antiaggregating therapy but venesection alone is often appropriate treatment for disease that is relatively indolent in activity.

Venesection preliminary to myelosuppressive therapy. The most effect of myelosuppressive therapy on the red cell count is not manifested for about three months.

Thus, it is advisable in most patients to reduce the total red cell volume by venesection to lessen the possibility of thrombosis, and relieve symptoms over this initial period.

Venesection is Indicated

a. When symptoms are distressing. Cerebral symptoms, e.g. headache, dizziness, and fullness in the head, respond particularly well to venesection;
b. In patients with a markedly increased PCV; and
c. In patients with a history of a previous thrombotic episode.

In other patients, venesection is optional, although advisable. Venesection of 300–500 mL is carried out either daily or on alternate days, until the PCV is about 0.55; this is usually achieved in 1–2 weeks. In patients with critical ischemia, it is advisable to replace each unit of blood removed with a high molecular weight dextran preparation to reduce blood viscosity by maintaining the plasma volume. Most patients suffer no ill effect from rapid venesection, but caution should be exercised with patient in whom cardiovascular symptoms are prominent. Particular caution is necessary in elderly subjects with a previous history of thrombosis, as hypotension which occasionally results from venesection may predispose to thrombosis, or ischemia; these hazards are largely obviated by replacement of blood with a dextran preparation. Following repeated venesections, red cell hypochromia often develops as a result of induction of iron deficiency. Administration of iron to subjects in whom iron deficiency has contributed to maintaining a low hemoglobin level and PCV can cause an increase in frequency with venesections are required to maintain an appropriate PCV.

Symptomatic Treatment

Patient with *hyperuricemia* may develop gout, renal colic, even oliguria from urate nephropathy; this is especially so in warmer climates. Thus they should have a high fluid intake with measures to alkalinize the urine, and be given the xanthine oxidase inhibitor allopurinol, in doses of 300 mg daily. After treatment has finished, in patient with hyperuricemia, allopurinol, should be given in a continuous daily dose of 100 or 200 mg. Some authorities recommend its use in all patients with polycythemia, on the ground that it may prevent the development of gout or renal calculi. *Pruritus*, which is thought to be due to histamine release, may be controlled by antihistamines, particularly cyproheptidine.

Hemorrhage and thrombosis occur frequently following surgery. Hemorrhage may be persistent, difficult to control, and is sometimes fatal; extensive wound hematomas are common. Comparison of cases controlled before surgery with uncontrolled cases revealed a threefold reduction on morbidity, and a sevenfold reduction in morality in the treated group. *Thus elective surgery should be brought under full hematological control over several months before surgery*. For emergency surgery, repeated venesection should be performed to bring the red cell volume to normal or near normal, with partial replacement by plasma if necessary to prevent circulatory collapse. At operation, special attention should be paid to local hemostasis. When hemorrhage does occur, platelet transfusion may be necessary if bleeding is judged to be due to platelet dysfunction.

FAMILIAL POLYCYTHEMIA (FAMILIAL ERYTHROCYTOSIS)

Polycythemia has been reported rarely as a familial condition; there is evidence to suggest transmission as a Mendelian dominant trait, but recessive inheritance has also been described. The condition presents at a younger age than classical polycythemia vera, often in childhood. There may be few, if any, symptoms, and leukocytosis and thrombocytosis

are absent. The prognosis appears to be relatively good, and for this reason the condition is sometimes called 'benign familial erythrocytosis'. Some of these families have been identified as possessing a high oxygen affinity hemoglobin; in others, a low erythrocyte 2, 3-DPG has been found; and in a third variety with recessive inheritance, inappropriately increased erythropoietin secretion has been identified. In others, no definite mechanism has been established.

RELATIVE POLYCYTHEMIA

In relative polycythemia, the total number of red cells in the body is not increased. The raised red cell count in the peripheral blood is due to contraction of the plasma volume, while the total red cell volume is normal. The diminished plasma volume may result from marked loss of body fluids, e.g. severe burns, dehydration, marked vomiting, persistent diarrhoea, diuretic therapy, and paralytic ileus. It can be due to reduced fluid intake or redistribution of body fluids, which can occur in crush injuries when the plasma passes into damaged tissues, as well as in other situations. Relative polycythemia due to actual bodily disturbance seldom presents any difficulty in diagnosis, as the cause of the hemoconcentration is usually obvious.

A further important category of relative polycythemia is now described under the heading of pseudopolycythemia.

PSEUDOPOLYCYTHEMIA

Polycythemia of 'stress' and spurious polycythemia are synonyms for pseudopolycythemia, which is a relative polycythemia of unknown etiology. It was called as 'polycythemia of stress' because about one-half of their patients had an anxiety state or were mildly neurotic, and it was thought that condition might be related to nervous stress. However, this condition is now known to be of mixed origin, partly relative polycythemia, and partly mild secondary erythrocytosis in heavy smokers due to inhalation of carbon monoxide.

Clinical picture: Pseudopolycythemia occurs much more commonly in males than in females, and although it may occur at any age in adult life, it is most frequently seen in middle-aged persons. There is no typical history. Symptoms of an anxiety state, e.g. fatigue, irritability, headache, and nervousness are common and some patients complain of dizziness. Hypertension or obesity is present in about 50% of cases. The facial complexion is florid, often being indistinguishable from that of polycythemia vera, and dilatation of the superficial vessels about the cheeks and nose is frequently present. The liver and spleen are not palpable.

Blood Picture

Red cell values are usually at about the upper limit of normal, or are slightly increased. Thus red cell counts of 6.5–7 $\times 10^{12}$/L, hemoglobin levels of 18–20 g/dL, and a PCV of 0.54–0.60 are usual, although both lower or higher values occur. The white cell and platelet counts are normal, as is the neutrophil alkaline phosphatase. The arterial oxygen saturation is normal. The aspirated bone marrow is of normal cellularity.

The total red cell column is normal. The increase in concentration of red cell is due to an abnormally low plasma volume, of unknown cause.

Diagnosis

The condition is of important because it may be confused with polycythemia vera and wrongly treated with myelosuppressive therapy. The main points of differentiation from

polycythemia vera are set out in Table given earlier. It is probable that many cases of Gaisboeck's syndrome are actually examples of pseudopolycythemia.

THROMBOCYTHEMIA

CAUSES OF RAISED PLATELET COUNT

Reactive

- Hemorrhage, trauma, postoperative
- Chronic iron deficiency
- Malignancy
- Chronic infections
- Connective tissue diseases, e.g. rheumatoid arthritis
- Postsplenectomy with continuing anemia

Endogenous

- Essential thrombocythemia
- In some cases of polycythemia vera, myelosclerosis and chronic myeloid leukemia

ESSENTIAL THROMBOCYTHEMIA (Fig. 15.3)

Megakaryocyte proliferation and overproduction of platelets is the dominant feature of this condition; there issustained increase in platelet count above 1000×10^9/L. The condition is closely related to polycythemia vera. Some cases show patchy myelosclerosis. Recurrent hemorrhage and thrombosis are the principal clinical features. Splenic enlargement is frequent in the early phase but splenic atrophy due to platelets blocking the splenic microcirculation is seen in 50% of patients.

There may be anemia (often due to iron deficiency from chronic gastrointestinal hemorrhage) or the thrombocythemia may be accompanied by polycythemia.

Laboratory Findings, Treatment, and Course

Abnormal large platelets and megakaryocyte fragments may be seen in the blood film. The condition must be distinguished from other causes of a raised platelet count. Platelet function tests are consistently abnormal. ^{32}P or alkylating agents are employed to reduce platelet production; the doses required are less than in polycythemia vera. Control of hemorrhage may lead to an increase in total red cell volume to polycythemic level.

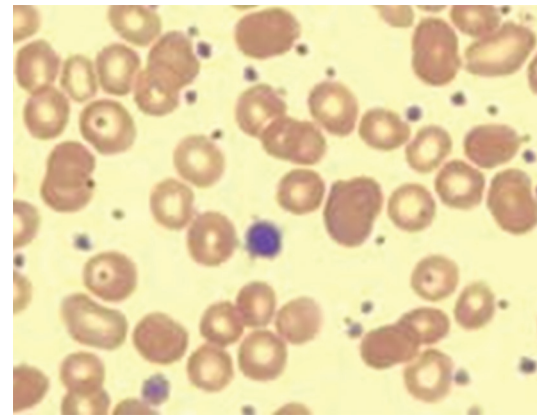


Fig. 15.3: Essential thrombocythemia, peripheral smear

MYELOFIBROSIS (MYELOSCLEROSIS)

Myelofibrosis is used to describe fibrosis and collagenisation in the marrow. The terms myelofibrosis and myelosclerosis have both been used to describe cases with collagen deposition and new bone formation, but the term myelofibrosis is now preferred. Myelofibrosis may be classified as primary or secondary.

Primary Myelofibrosis

Myelofibrosis developing in polycythemia vera may be considered as a variant of this disorder. It is probable that in about 25% of cases of primary myelofibrosis there is a preceding history of polycythemia vera.

Secondary Myelofibrosis

Develops in association with some well-defined disorder of the marrow, or as a result of the toxic action of chemical agents or irradiation. Thus, fibrosis may develop in association with tuberculosis, secondary carcinoma, Hodgkin's diseases, leukemia, and a variety of other hematological disorders.

Primary myelofibrosis is usually accompanied by myeloid metaplasia (extramedullary hemopoiests) in the spleen and liver, and to a much lesser extent in the kidney, lymph nodes, and other organs. The myeloid metaplasia involves the white and red cell precursors and megakaryocytes. In primary myelofibrosis, myeloid metaplasia is consistently found, but in the secondary form it is much less common. Occasionally, myeloid metaplasia is found at postmortem as tumour masses in various organs.

PRIMARY MYELOFIBROSIS

Primary myelofibrosis is also known as myelosclerosis and agnogenic myeloid metaplasia, which is a proliferative neoplastic disorder related to polycythemia vera and essential thrombocythemia. The term myeloproliferative disorder is sometimes applied to this group as a whole. Intermediate or transitional forms of these disorders, showing overlapping clinical and pathological features, are seen. Thus myelofibrosis commonly develops in the terminal phase of polycythemia vera.

The blood picture of acute myeloid leukemia not uncommonly develops late in the disorder. This may be the terminal/terminating illness in many such cases (Fig. 15.4).

The myeloproliferative disorders are due to uncontrolled proliferation of abnormal progenitor cells which are capable of producing erythroid, myeloid, and megakaryocytic series. The particular type of disorder is determined by the predominant series into which the cell differentiates, and the extent to which hemopoiesis is 'effective' or 'ineffective'.

In polycythemia vera, the proliferation predominantly involves erythropoiesis and the 'effective', giving rise to increased red cell production and high red cell counts. Similarly, where megakaryocyte proliferation is predominantly involved and thrombopoiesis is 'effective', essential thrombocythemia results. Some cases with 'panmyelosis' have high blood count of all three cell forms, as seen in florid polycythemia vera. As the diseases evolves, hemopoiesis frequently becomes 'ineffective', and blood cell count falls. Products of the cells are released in the marrow, including the platelets-derived cell growth factor from megakaryocytes and stimulate deposition of reticulin and fibrous tissue. Products from other cells may also play a part. This view is supported by evidence that fibroblasts in myelofibrosis are not derived from the same clone as the abnormal hemopoietic cells but are relative normal cells. Megakaryocytic hyperplasia

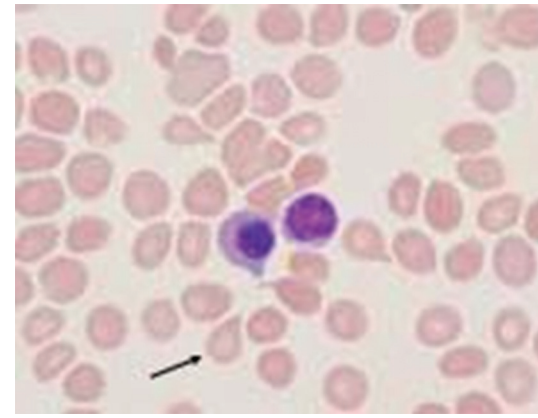


Fig. 15.4: Myelofibrosis, peripheral smear

is often prominent in the marrow in myelofibrosis, even though platelet production may be ineffective.

Earlier, the myeloid metaplasia in the spleen, liver, and other organs in myelofibrosis was thought to be a compensatory process to make up for the loss of normal blood-forming marrow. However, it is now considered to represent another manifestation of tissue infiltration by the primary disorder. Support for this theory is derived from the fact that in polycythemia vera, the histology of the liver and spleen shows that myeloid metaplasia occurs while the bone marrow is still hyperplastic and the blood polycythemic before the onset of myelofibrosis. Deposition of reticulin and collagen also commences in the marrow at this stage of the disease.

Clinical Picture

Myelofibrosis is usually a disease of adult life, occurring most commonly between the ages of 40 and 70 years; rarely it occurs in young adults, and even children. It appears to occur equally in both sexes.

The onset is insidious, the condition usually being present for some time before the diagnosis is obvious. The patient most frequently presents with symptoms of anemia, especially weakness, or with symptoms due to splenomegaly. The accidental finding of an enlarged spleen, either by the patient or doctor, is sometimes the first indication of the disease. Occasionally, weight loss, anorexia, bleeding manifestations, acute abdominal pain, gout, bone pain, leg cramps, or jaundice are presenting manifestation. The development of myelofibrosis in patients with polycythemia vera is accompained by a fall in the hemoglobin level, together with relatively rapid enlargement of the spleen.

The symptoms of the anemia are those common to all anemias, namely weakness, lassitude, fatigue, dyspnea on exertion, and palpitation. With severe anemia, signs of congestive cardiac failure may develop.

Splenomegaly

Splenomegaly is the outstanding physical sign, the spleen usually extends below the umbilicus, and in the later stages may be grossly enlarged, extending into the left iliac fossa, and sometimes appearing to fill the whole abdomen. Slow progressive enlargement of the spleen over many years can often be observed, although in acute myelofibrosis the spleen may not be greatly enlarged. Symptoms due to splenomegaly are common, e.g. abdominal fullness, or a dragging, aching sensation, or pain in the left hypochondrium. When the spleen is very large, epigastric discomfort after meals, flatulence, dyspepsia, nausea, and frequency of micturition may occur. Splenic infarction is common, causing acute pain and sometimes a splenic friction rub.

Hepatomegaly

Hepatomegaly is common. Enlargement is usually slight to moderate, although occasionally the liver extends below the umbilicus. The liver is firm, smooth, and non-tender. Following splenectomy, the liver may rapidly increase in the size due to an increase in myeloid metaplasia. Mild jaundice is common, especially in the latter stages, and is mostly unconjugated bilirubin produced by ineffective erythropoiesis. Portal hypertension with associated features such as esophageal varices occurs in up to about one-quarter of cases of myelofibrosis.

Lymph Node Enlargement

Lymph node enlargement is unusual in the typical disorder, and when present is only slight.

Constitutional Symptoms

Weight loss, wasting, weakness, and lassitude out of proportion to the degree of anemia commonly develop in the later stages of the disease, and are sometimes present early. Less commonly, night sweats occur, and pruritus is occasionally present, especially in cases evolving from polycythemia vera.

Bleeding Manifestations

Bleeding manifestations are common, especially in the latter stages. Bleeding is usually due to thrombocytopenia, purpura and epistaxis being particularly prominent. However, bleeding, especially from the gastrointestinal tract may occur in patients with normal platelet counts. The gastrointestinal bleeding is sometimes due to peptic ulceration which is more common than in the general population, and sometimes from esophageal varices.

The serum uric acid is commonly raised, and gout occurs; it may be exacerbated by splenic irradiation or myelosuppressive therapy. Vague bone pains, particularly in the legs, are not uncommon, and occasionally bone tenderness, especially of the sternum, is present. However, in general, the presence of marked bone pain or tenderness in a patient with leukoerythroblastic anemia suggests a cause other than myelosclerosis, such as secondary carcinoma in bone or acute leukemia.

Radiological Bone Changes

Radiological bone changes occur in about 30 percent of cases, and are seen especially in the later stages. The changes are usually not marked, and are best demonstrated by comparison with X-rays of normal persons of similar age. The typical picture is one of patchy sclerosis of the medullary cavity, often with coarsening of trabeculation and rarefaction, sometimes giving a mottled appearance. The bones most commonly involves are the pelvis, spine, and upper ends of the femora and humeri.

Blood Picture

The classical blood picture is that of a leuco-erythroblastic anemia with marked anisocytosis and poikilocytosis. The white cell and platelet counts vary; they may be normal, raised, or reduced. Thus, occasionally, the blood picture is of pancytopenia.

Anemia is almost invariable at some stage in the course of the disorder, but its severity and the rate of progress vary considerably. In many cases, it is of the slight to moderate degree at the time of diagnosis, and remain relatively constant over a number of years. However, in the later stages of disorder anemia usually becomes more severe. Sometimes, the hemoglobin level is within normal range at the time of diagnosis, particularly when the disorder has evolved from polycythemia vera. Three main factors contribute to the anemia, namely impairment of red cell production, pooling of red cells in the spleen, and hemolysis. Defective erythropoiesis is usually the major factor, but increased plasma volume associated with the splenomegaly may also contribute to the anemia.

RBCs

The RBCs are usually normocytic and normochromic, but anisocytosis is often marked. Iron deficiency may develop, in which case microcytosis and hypochromia are found. If macrocytosis is present, it may indicate complicating folate deficiency. Moderate to marked poikilocytosis is usual, pear-or tear-shaped poikilocytes being especially characterisitic of the disease; oval or elliptical cells are also common. Polychromasia and basophilla are often prominent, and the reticulocyte count may be moderately raised. Some fragmented

and spherocytic cells may be present, especially in the later stages. Nucleated red cells are most invariably present, usually in small, but sometimes in large numbers, when they constitute a striking feature of the blood film; they are orthochromatic or late polychromatic, although earlier forms are sometimes seen. Commonly ther are up to 10 normoblasts per 100 white cells, but occasionally they form a great percentage, especially when the total white cell count is low. The number of normoblasts does not parallel the degree of anemia, and they may be numerous even when anemia is slight.

White Cell Count

The total white cell count is usually normal or moderately raised, but it is sometimes reduced. When raised, the count is usually not more than 50×10^9/L, but occasionally it is higher, and rarely it exceeds 100×10^9/L, mostly after splenectomy. When reduced, counts range from about 2–4 $\times 10^9$/L. Myelocytes and metamyelocytes are usually present, but are rarely very numerous, and together commonly comprise 10–20% of the total white cells, although higher propositions may occur. A small number of promyelocytes and myeloblasts may also be seen. There is sometimes a slight increase in the proportion of eosinophils and basophils. The alkaline phosphatase content of the granulocytes is usually increased, but may be normal or rarely decreased.

The *platelet count* is usually normal or reduced, but may be raised, sometimes to more than 1000×10^9/L. Thrombocytopenia most commonly occurs in the later stages, when it may be seen, and megakaryocytic fragments are sometimes present. A number of abnormalities of platelet function occur.

A moderate increase in the ESR is common. A moderate rise in serum bilirubin, e.g. 30–50 mmol/L, is not uncommon; this is mostly unconjugated. The serum lactate dehydrogenase and lysozyme levels are elevated, reflecting the increased but ineffective hemopoiesis that is taking place.

Serum folate values are commonly reduced because of the increase in folate requirements produced by the utilization of folate by the abnormal tissue. Often, red cell folate values are reduced; in one series, megaloblastic hemopoiesis due to folate deficiency occurred at some time during the course of disease in one-third of patients with myelofibrosis. This point is of therapeutic importance in the management of anemia in this condition. Folate deficiency sometimes becomes obvious after an infection. The serum vitamin B_{12} is normal or elevated.

Bone Marrow

The main pathological feature is proliferation of fibroblasts, resulting in a diffuse increase of reticulin fibers and deposition of collagen in the narrow, often with thickening of the bony trabeculae which encroach on the marrow cavity. The degree of fibrosis varies from patients to patient, and often in the same patient at different sites. The increase in the reticulin fibers is shown by the reticulin stain and in the early stages this may be the only significant evidence of fibrosis, with little or no collagen formation. Areas of active hemopoietic tissue are found scattered in the fibrotic tissue. Megakaryocytes are often present in markedly increased numbers; however, there is no correlation between their number and the peripheral platelet count.

Marrow aspiration is by and large unsatisfactory, usually resulting in a ‘dry’ tap. Less commonly, aspiration yields a small amount of blood with a few marrow cells, a number of which are megakaryocytes or megakaryocytic nuclei. A gritty sensation is sometimes felt when the needle enters the marrow cavity. Occasionally, the needle enters one of the

areas of active marrow interspersed in the fibrotic tissue, and marrow fragments of either normal or increased cellularity are aspirated.

Marrow trephine biopsy, preferably of the iliac crest, usually yields a satisfactory sample containing the characteristic histological features, which establish the diagnosis. The histological findings on biopsy are the variability of the degree of fibrosis and the extent of splenomegaly. An increase in reticulin fibers alone is not in itself diagnostic of primary myelofibrosis, as this may occur in other marrow disorder such as chronic granulocytic leukemia and other neoplastic diseases.

Diagnosis

The diagnosis is assisted by the occurrence of marked splenomegaly with leucoerythroblastic anemia, often with relatively little deterioration in general health, in a middle-aged or elderly person. Sometimes, the spleen is known to have been enlarged for a number of years. Cases evolving from polycythemia vera often have a previous history of symptoms suggesting antecedent polycythemia, e.g. a plethoric appearance, bloodshot eyes, or pruritus. Failure of marrow aspiration at more than one site further suggests, but does not confirm, the diagnosis. Marrow trephine is essential for diagnosis.

Diagnostic difficulty may occur when, on marrow aspiration or trephine, the needle enters one of the areas of active marrow interspersed in the fibrotic tissue, and marrow of either normal or increased cellularity is obtained. In such cases, marrow examination at another site usually reveals the typical findings.

Myelofibrosis associated with tuberculosis: Rare cases of myelofibrosis have been described in association with, and thought to be due to, disseminated tuberculosis. Although the peripheral blood picture may resemble that of myelofibrosis, the clinical picture shows certain general differences—it can occur in younger patients; fever, malaise, and other manifestations of tuberculous toxemia are present; splenomegaly is less marked; moderate generalized lymph node enlargement is usual; and the course is shorter. The diagnosis is established with certainty only if the marrow biopsy specimen includes a tuberculous focus.

Differential Diagnosis

Other Causes of Splenomegaly

The major problem in the diagnosis of primary myelofibrosis is differentiation from chronic granulocytic leukemia, with which it is commonly confused, as patients with both conditions can possess marked splenomegaly and immature granulocytes in the peripheral blood. Fibrosis can occur in the marrow in chronic granulocytic leukemia, and the main differential points are listed in the table given earlier.

Disorders with a Similar Blood Picture

In most cases, careful consideration of the clinical and hematological features and special investigations will distinguish these disorders:

Other Causes of Leukoerythroblastic Anemia

Macrocytic anemia

When macrocytosis is prominent, the association of macrocytosis with marked anisocytosis and poikilocytosis, espically when the white count is low, may suggest megaloblastic anemia. In most cases, the spleen is much larger and firmer than ever occurs in pernicious

anemia or other megaloblastic anemias; further, the presence of more than occasional myelocytes suggests myelofibrosis.

Hemolytic anemias

The occurrence of polychromasia, reticulocytosis, normoblastemia, and splenomegaly, especially when jaundice is present, may suggest a diagnosis of hemolytic anemia. In hemoytic anemia, however, the evidence of dyshemopoiesis, namely anisocytosis and poikilocytosis, is seldom so great.

Other Causes of Bone Marrow Fibrosis

It has been observed that bone marrow involvement occurring in certain disorders may be accompanied by fibrosis. These include secondary carcinoma, tuberculosis, malignant lymphomas, and leukemia. Usually, diagnosis of these disorders is obvious from the clinical features and special investigations, and the marrow fibrosis is simply an incidental finding. In particular, the rate of development of anemia is usually more rapid in these disorders than in myelofibrosis. As marrow aspiration may result in a 'dry' tap under a variety of circumstances, trephine biopsy is essential. The demonstration of fibrosis does not, on its own, make the diagnosis, and if the other expected clinical and hematological features are not present, other causes of marrow fibrosis must be carefully considered.

Course and Prognosis

The course of primary myelofibrosis is usually chronic, but is occasionally relatively rapid. The average duration of life is 5–7 years from diagnosis, but individual survivals vary from 1 to more than 20 years. Survival for 10 years or longer is not uncommon. The anemia is often only slight to moderate and remains relatively constant for a number of years, with the result that there is little interference with general health even though splenic enlargement maybe considerable. Eventually, anemia becomes severe enough to require transfusion. After repeated transfusions, the increase in hemoglobin level becomes less and is of shorter duration, until it is often impossible to control the anemia by transfusion. In other cases, the anemia initially is more severe and progresses more rapidly, with the result that the disorder can be fatal within one year of diagnosis. Unfavorable prognostic signs are severe anemia responding poorly to transfusion, severe leukopenia, spontaneous bleeding, especially when due to thrombocytopenia, and marked weight loss. Ineffective hemopoiesis may be associated with severe hyperuricemia, which may prove difficult to control. Death commonly occurs from anemia, cardiac failure, bleeding, or intercurrent infection. About one-quarter of cases terminate as actual myeloid leukemia.

Treatment

No specific therapy exists, treatment being primarily symptomatic. However, many patients initially have only mild symptoms and require little or no treatment for the years following diagnosis. Anemia and the discomfort due to splenomegaly are the main symptoms requiring treatment, but treatment of hemorrhage may be necessary. Folic acid, androgens and in, the later stages, blood transfusion are the main forms of treatment for anemia. Iron deficiency should be watched for and treated when present.

Discomfort due to Splenomegaly

Discomfort due to splenomegaly can sometimes be lessened by the wearing of a supportive abdominal belt. Busulfan may be helpful in reducing splenomegaly, and is particularly useful when the granulocyte count is high. In selected cases, splenic irradiation gives

temporary relief, but the reduction in spleen size with radiation in this disease is less than in chronic granulocytic anemia.

Hemorrhage

When hemorrhage is due to thrombocytopenia, splenectomy may be considered as an approach to elevating the platelet count. However, in general, severe thrombocytopenic bleeding occurs in patient with peptic ulceration and a high platelet count, reduction of the count to normal by the use of busulfan may lessen the liability to bleed. In some patients, on the other hand, a high platelet count may persist for many years without complications.

Hyperuricemia

Hyperuricemia, with its sequelae of gout, nephrolithiasis, and urate nephropathy, usually responds to allopurinol, which is especially important in preventing a further rise in the serum uric acid following treatment with busulfan or splenic irradiation.

Folic Acid

Because of the relatively high incidence of folate deficiency in this disorder, folate deficiency must be excluded as a contributing factor in the development of anemia or thrombocytopenia. Before any other treatment is given, a serum folate (and red cell folate) estimation should be performed. If values are low, a therapeutic trial of folic acid should be given, and this sometimes eliminates the need for transfusion.

Androgens

Administration of androgens such as oxymetholon should be considered for patients with symptoms of anemia, as a significant minority undergo a degree of improvement. Doses of 100–200 mg daily are usually employed, and responses tend to be delayed for at least 6 weeks, treatment should be ceased, as it is less likely to be beneficial. When response does occur, continued administration is usually necessary to maintain the increased hemoglobin level, but sometimes improvement is sustained after cessation of treatment. Less than one-half of patients obtain some benefit, and those with chromosomal abnormalities are reported to be less likely to respond.

An initial trial of testosterone enanthate, 600 mg intramuscular weekly for 6 weeks, this can then be slowly reduced to a dose necessary to maintain a satisfactory hemoglobin level. This approach remains useful for male patients who do not bruise. Oxymetholone, the anabolic agent most widely used for treatment of aplastic anemia, is now probably the agent of choice.

Blood Transfusion

In patient with slight anemia, transfusion may not be required for many years. When anemia is sufficiently marked to require transfusion, transfusions should be kept to the minimum necessary to maintain the hemoglobin at a level consistent with a comfortable active life; this is usually about 9 g/dL. Even with the initial transfusion, the rise in hemoglobin is often less than expected for the amount of blood given, but nevertheless a significant rise, followed by a slow fall over a period of weeks or months, is usual. With repeated transfusions, the rise in hemoglobin usually becomes less and of shorter duration, until eventually a point is reached when even large transfusions may give a response lasting only a week or two, or even less. The cause for this progressive ineffectiveness of

transfusion is variable. In some cases, the development of immune isoantibodies play a part, but in many cases sequestration of the transfused cells in the spleen is the major problem. When transfusion is no longer able to adequately control the anemia, the administration of corticosteroids should be tried, and if this does not result in a significant lessening of transfusion requirements, splenectomy should be considered. This usual precaution essential in the transfusion of patients with chronic anemia must be carefully observed.

Busulfan, Hydroxyurea

Busulfan, hydroxyurea, or other myelosuppressive therapy can be considered in patients with high leukocyte or platelet count, relatively cellular marrow, and a large spleen when there are overt problems related to progressive disease. The dosages used are as for the treatment of polycythemia vera, and the response must be carefully monitored and the drug withdrawn if leukopenia is produced.

Corticosteroids

In general have little to offer, but in patients with increasing transfusion requirements in whom it is difficult to maintain the hemoglobin level, corticosteroid treatment maybe tried. Prednisolone is given initially in doses of 25–75 mg daily for 2–3 weeks, followed by progressive dose reduction, and sometimes results in a lessening of transfusion requirements, together with some increase in hemoglobin level and slight reduction in splenic size. However, in general, the response is disappointing, and if ineffective, treatment should be ceased.

Splenectomy

Usually, splenectomy has nothing to offer and is associated with perioperative morbidity and mortality. However, in selected cases, especially in the latter stages of the disease, it may be beneficial. It should be considered:

a. When transfusion requirements have increased to such a degree that it is difficult or impossible to maintain the hemoglobin at a comfortable level. In such cases, splenectomy sometimes lessens transfusion requirements.
b. When thrombocytopenia is sufficiently severe to cause troublesome bleeding, as splenectomy is often followed by a rise in platelet count, and
c. When the spleen, because of its massive size, causes pressure symptoms.

However, splenectomy has several disadvantages; it has a relatively high operative mortality and morbidity, because of the bleeding tendency and, in the later stages, the poor general condition of the patient. In addition, marked elevation of the platelet count sometimes follows splenectomy, particularly in patients with normal or raised pre-splenectomy count. This predisposes to thrombosis. If significant thrombocytosis persists after splenectomy , alkylating agents or hydroxyurea should be used to reduce the platelet count. In some patients, marked enlargement of the liver occurs rapidly after splenectomy, causing abdominal discomfort and sometimes, recurrence of anemia or thrombocytopenia.

Thus the possible benefit of splenectomy must be carefully weighed against the risks. However, it is important that splenectomy, if indicated, should not be left too late, as seriously ill patients often do not survive the operation.

Splenic Irradiation

Splenic irradiation does not favourably affect the course of the disease, and in most cases is not used. However, it may be of value in patients with severe pressure symptoms associated

with marked splenomegaly. In such cases, irradiation may be followed by reduction in splenic size, with relief of pressure symptoms and of constitutional symptoms, especially when the leukocyte count is raised. The total white cell count falls, as does the number of immature white and red cells. The dose of X-ray should be minimum necessary to relieve the symptoms. The complications of hyperuricemia may occur, and thus allopurinol should be given. The first course is the most effective. Irradiation is relatively dangerous in patients with low white counts. The benefit is often short lived, a return to previous splenic size within several months being common.

ACUTE MYELOFIBROSIS

A variant of myelofibrosis has been recognized which runs an acute course and which has clinical and hematological features sufficiently distinctive to require separate classification. It occurs most commonly in middle-aged and elderly subjects. It differs in that splenomegaly is often absent or of relatively minor degree.

The blood picture typically is of pancytopenia, often with profound neutropenia and the presence of blast cells and myelocytes. Nucleated red cells may or may not be present. Marrow trephine usually reveals a cellular marrow with an increase in reticulin, often without a marked increase in collagen. These cases represent malignant proliferation of megakaryoblastic series and overlap into frank megakaryoblastic (M7) leukemia

Intensive remission-induction therapy, as for acute leukemia, can be considered, but results are generally disappointing, and occasional beneficial responses have been obtained with low dosage cytarabine. These is a significant pyrexia without demonstrable infection in some cases, and the condition must be distinguished from poorly differentiated non-Hodgkin's lymphoma with bone marrow involvement, which may produce a similar clinical picture.

CAUSES OF MARROW FIBROSIS

- Myelosclerosis
- Infections
  - Tuberculosis
  - Osteomyelitis
- Malignant lymphoma
  - Including Hodgkin's disease
- Occasionally in chronic myeloid and other leukemia
- Metastatic carcinoma
  - Especially breast and prostatic carcinoma
- Excess irradiation
- Benzene poisoning
- Excess fluorine
- Paget's disease
  - Focal fibrosis
- Osteopetrosis

CHAPTER

16

Platelets, Blood Coagulation, and Hemostasis

BLEEDING DISORDERS

The bleeding disorders are a group of disorders of widely varying etiology, which have in common an abnormal tendency to bleed due to a defect in the mechanism of hemostasis.

CLINICAL PICTURE

Clinically, the hemorrhagic disorders are characterized by:

a. Spontaneous bleeding into the skin, mucous membranes and internal tissues
b. Excessive or prolonged bleeding following trauma or surgery; and
c. Bleeding from more than one site.

The bleeding varies in severity. In some disorders, it is mild and limited to the skin and is therefore of nuisance value only; in other disorders, uncontrollable bleeding from mucous membranes or bleeding into internal organs may threaten life.

PATHOGENESIS

There are three major components of the normal hemostatic mechanism–the vascular, platelet, and coagulation components—which act together in a coordinated fashion to arrest bleeding. A breakdown in the normal hemostatic mechanism, and thus an abnormal tendency to bleed, may result from a defect in any one of these three components. Bleeding is especially liable to occur when more than one component is defective. It should be noted that bleeding may arise from either quantitative (deficiency) or qualitative (functional) defects.

In this section, hemorrhagic disorders due to vascular and platelet abnormalities are discussed. Coagulation defects are discussed in Chapter 17: Coagulation Disorders.

NORMAL HEMOSTATIC MECHANISM (FIG. 16.1)

The functions of the normal hemostatic process are to prevent blood loss from intact vessels and to arrest bleeding from injured vessels. Relatively little is known about the mechanism by which blood loss is prevented from intact vessels; however, both the structural integrity of the vessels and the presence of adequate numbers of normal viable platelets are necessary for this function. The precise nature of the functional support of the endothelium provided by platelets is not known, but there is evidence for structural changes in the vessel wall as a consequence of thrombocytopenia both in experimental animals and in humans. Although there has been controversy surrounding the question, there appear to be growth factors within platelets which may be involved in stimulating endothelial repair process.

Immediately after injury the damaged blood vessel undergoes a temporary reflex nervous vasoconstriction, resulting in slowing of blood flow. Blood escapes into the tissues and so increases the tissue tension, with further narrowing of the vessels (see extravascular factors below). The escaping blood comes in contact with the damaged vessel wall and the extravascular tissues, and the processes of platelet adhesion, platelet aggregation, and blood coagulation are initiated. The response to an endothelial breach is extremely rapid; the time available for a platelet to leave the blood flow and react to the subendothelium may be measured in milliseconds.

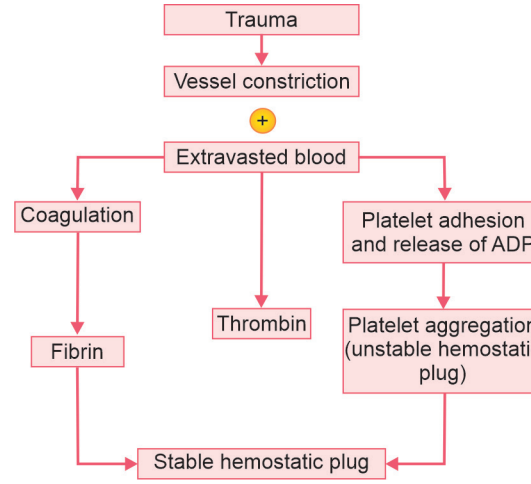


Fig. 16.1: Normal hemostasis

Platelet adhesion to subendothelium has two definable components, each with its own functional determinants. The initial contact adhesion is to types I and III collagen fibrils and to the elastin-associated collagenous microfibrils in subendothelium. At high shear flow (>800 sec^{-1}) in the capillary bed, platelet adhesion to these subendothelial components depends on the plasma protein, von Willebrand factor (factor VIII-related antigen) which forms a bridge to the platelet membrane receptor, glycoprotein Ib complex. In larger vessels, e.g. the aorta, platelet adhesion is independent of von Willebrand factor and probably involves the direct adhesion of platelets to collagen fibrils. This may be via the platelet membrane glycoprotein IIb/IIIa complex and glycoprotein Ia (Fig. 16.2).

Platelet contact is closely followed by a process of spreading adhesion dependent on at least three components:

a. Adhesive proteins such as von Willebrand factor and/or fibronectin incorporated in the subendothelial matrix
b. Divalent cations, calcium or magnesium, and
c. The glycoprotein IIb/IIIa complex in the platelet membrane.

Release of platelet components and the recruitment of further platelets occur simultaneously, but can be conveniently considered as two distinct events. Both are stimulated by the adhesion process to collagen and by a number of other agonists made available in the vicinity of tissue injury, particularly adenosine diphosphate (ADP) and thrombin, and, in some situations, platelet activating factor (PAF) derived from inflammatory cells. There are also common internal pathways within the platelet, which initiate the processes of release and aggregation. The

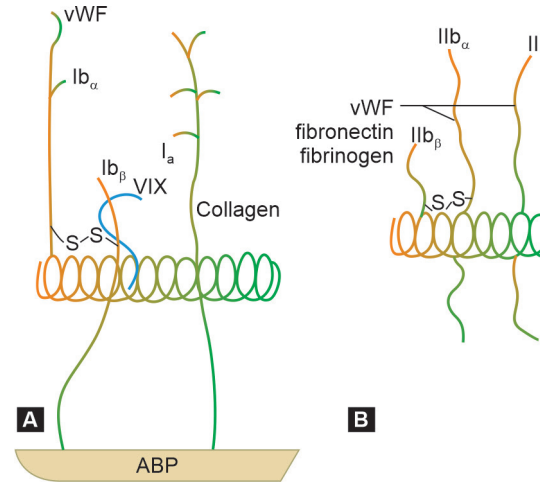


Fig. 16.2: Diagrammatic representation of the structures of the human platelet glycoprotein: (A) Ib complex and Ia; (B) IIb/IIIa complex. vWF = von Willebrand factor; ABP = Actin binding protein.

signal transduction of receptor occupation involves the hydrolysis of phospho-inositol by phospholipase C; hydrolysis of phosphatidylcholine and phosphatidylethanolamine by phospholipase A, with release of arachidonic acid; the activation of protein kinase C; and the ultimate increase of cytosol [Ca^{++}] released from the dense tubular system and by influx from the outside.

Aggregation is dependent (Fig. 16.3)

a. Firstly, on shape change involving the loss of the platelet discoid configuration, and
b. The formation of pseudopods to increase the surface area and the likelihood of contact.

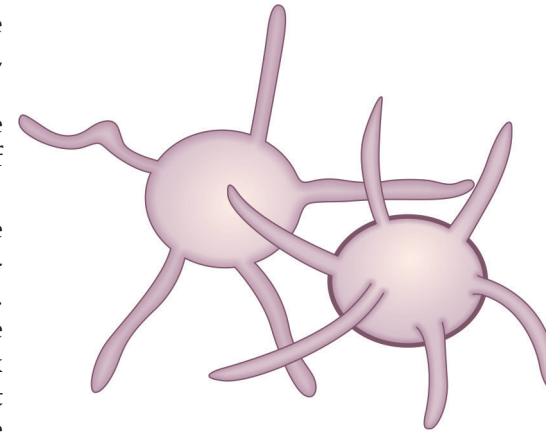


Fig. 16.3: Shape change and aggregation of platelets. Features of activation include the change to spherical shape, formation of pseudopodia, centralization of granules, and close contact with contiguous platelets

Shape change and the later release process depend on contraction of actin-myosin in the periphery of the platelet. Associated conformational changes to the membrane glycoprotein IIb/IIIa complex expose fibrinogen receptors on the platelet surface, enabling platelet bridging to take place via fibrinogen molecules, probably being enchanced by von Willebrand factor and fibronectin. The release of dense granule components, ADP, calcium, and serotonin, combined with the synthesis and release of thromboxane A_2 (TXA_2) from arachidonic acid, provides a powerful amplification feedback to aggregate platelets in the vicinity. The release is achieved by a combination of fusion and extrusion of granules.

Alpha granule proteins, factor V, fibrinogen, von Willebrand factor, and thrombospondin, released simultaneously, may contribute further to the process by raising their local concentration. Other alpha granule proteins and arachidonate products produced simultaneously are involved in repair processes following injury. Many alpha granule components are well characterized and include polypeptides such as the beta-thromboglobulin family (chemotaxis and possibly mitogenesis), platelet factor IV (antiheparin, chemotaxis) and platelet derived growth factor (chemotaxis, mitogenesis, and vasoactivity). Lipid-derived mediators such as 12-hydroxyeicosatetranoic acid (12-HETE), a lipoxygenase product of released membrane arachidonic acid have powerful chemotactic activity for neutrophils. Lyzosomal enzymes, released separately, include glucuronidase, n-acetyl glucosaminidase, elastase, heparinitase, and collagenase. These enzymes may reach local concentrations high enough to disrupt subendothelial basement membrane and internal elastic lamina (Fig 16.4).

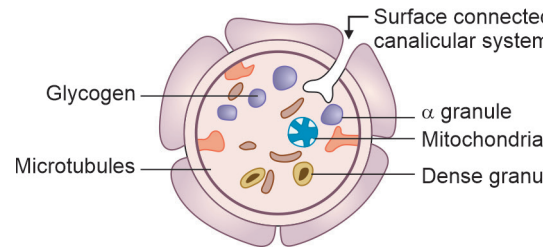


Fig. 16.4: Ultrastructural features of the platelet. Granule contents either fuse with the membrane or with the surface-connected open canalicular system before being extruded from the cell

Also in the process of aggregation, the platelet membrane exposes a number of receptor sites for coagulation components

making a 'solid phase' system for coagulation components mechanism, in part protected from inhibitors. The negatively charged phospholipid component that enhances coagulation enzyme systems, previously termed 'platelet factor 3', lies on the inner aspect of the platelet membrane but is exposed during activation, providing a surface for calcium and activated coagulation factor complexes. Factor V is released from alpha granules, activated by protease, and binds to specific sites on the platelet membrane. Membrane-bound factor Va becomes a receptor for activated factor X, accelerating the activation of prothrombin to thrombin some 300,000-fold.

Importance of various factors concerned with hemostasis varies with the size of the vessel involved. In small arterioles and venules, hemostasis depends mainly on vessel constriction and on a platelet plug. In larger vessels, although constriction is important in limiting the initial loss of blood, the formation of the hemostatic plug to seal the defect in the vessel plays an important major role.

Lastly limitation of the processes of platelet adhesion and activation is obviously of importance in maintaining blood fluidity and vascular patency.

The aggregating compounds have extremely short survival times due to hydrolysis (TXA2), enzymatic degradation (ATP and ADP), cellular uptake (serotonin), and specific inhibitors (antithrombin). Also, vascular endothelial responses to local platelet activation include the release of specific platelet inhibitory substances, in particular prostacyclin, a cyclooxygenase product of arachidonic acid (Fig. 16.5).

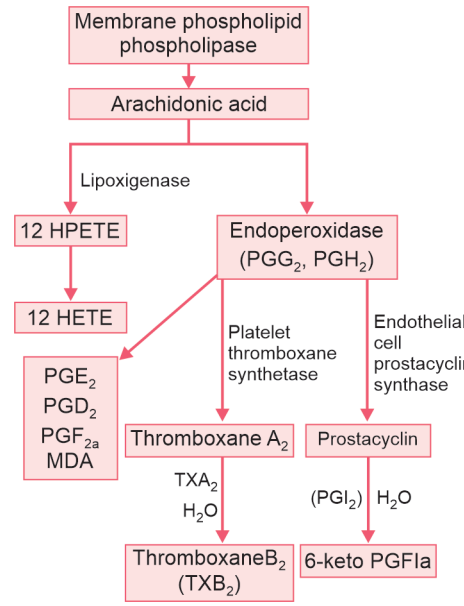


Fig. 16.5: Arachidonic acid metabolism in platelets and vascular endothelium. Thromboxane A_2 and prostacyclin are short-lived intermediates whose end-products, thromboxane B_2 and 6-keto-prostaglandin $F_{1}\alpha_1$ are stable and may be measured by radioimmunoassay. HPETE = Hydroperoxyeicosatetranoic acid; HETE = Hydroxyeicosatetranoic acid; MDA = Malondialdehyde

HEMOSTATIC PLUG—EVENTUAL FATE

The platelets in the hemostatic plug gradually undergo autolysis and are replaced by fibrin, so that after 24-48 hours a hemostatic plug has been transformed into a dense fibrin mass. This is then gradually digested by the fibrinolytic enzyme system and the defect in the vessel wall then becomes covered with endothelial cells.

EXTRAVASCULAR FACTORS

Extravascular factors also influence hemostasis by, namely tissue tension and the support of the vessels, which play an important subsidiary role, particularly in venous bleeding. In tissues with a relatively high tissue tension, the natural tension, together with the increased tension caused by the mass of escaped blood, compresses these damaged vessels and lessens blood loss. When vessels are contained in loose tissue and are poorly supported, bleeding tends to continue. Thus, vessels in the nasal septum, which have a rigid unyielding septum on one side and no support on the other, are particularly liable

to bleed. Vessels in the gastrointestinal mucosa, in the bladder, and in the pelvis of the kidney are not well supported; bleeding from these sites occurs relatively easily after slight trauma and in disorders of the hemostatic mechanism, and can be difficult to control. In other areas where the tissue tension is low, e.g. in the subcutaneous tissues of the scrotum and about the orbit, mild trauma may result in extensive hematoma formation.

HEMORRHAGIC DISORDERS DUE TO CAPILLARY DEFECTS: NONTHROMBOCYTOPENIC PURPURA (TABLE 16.1)

Defects of blood vessels are a common cause of bleeding disorders seen in clinical practice. However, it is now understood that in a number of the acquired vascular hemorrhagic disorders qualitative platelet defects may coexist and contribute.

Usually cases of bleeding due to a vascular defect alone are not severe, and frequently the bleeding is mainly or wholly into the skin, causing petechiae or ecchymoses, or both. Petechiae may be rather pale and tend to be confluent; ecchymoses are usually small. In some disorders there is bleeding from mucous membranes, but only rarely is there bleeding into muscles and internal organs. Excess bleeding from wounds tends to occur at once, usually persists for less than 48 hours, and rarely recurs.

In many of these conditions, the standard screening tests used in the investigation of patients with a bleeding disorder show little or no abnormality. The bleeding time is sometimes prolonged, and the tourniquet test may be positive. However, in many cases either one or both are normal. When they are abnormal, and especially when the bleeding time is prolonged, bleeding is likely to be more severe, particularly following trauma and with surgery. The platelet count, the one-stage prothrombin time, and the activiated partial thromboplastin time are typically normal. However, in certain disorder, e.g. infection and uremia, associated thrombocytopenia may contribute to bleeding. In addition, in those disorders with platelet functional defects, especially uremia and dysproteinemia, there are abnormalities in the tests of platelet adhesion or aggregation.

Table 16.1: Hemorrhagic disorders due to vascular defects

- Acquired
- Simple easy bruising ('devil's pinches')*
- Senile purpura
- The symptomatic vascular (nonthrombocytopenic)
  - Purpuras
  - Uremia*
  - Cushing's disease and adrenocorticosteroid administration
  - Scurvy*
  - Dysproteinemias*—cryoglobulinemia, benign purpura hyperglobulinemia, macroglobulinemia, multiple myeloma.
  - Henoch–Schöenlein syndrome (anaphylactoid purpura)
- Miscellaneous disorders
  - Orthostatic purpuras
- Mechanical purpura
- Fat embolism
- Autoerythrocyte sensitization
- Systemic disorders—collagen disease, especially polyarteritis nodosa, amyloidosis, allergy
- *Congenital*
  - Hereditary hemorrhagic telangiectasis (Osler–Rendu–Weber disease)
  - Enhlers–Danlos disease

*Abnormalities of platelet function may contribute to the bleeding tendency in these disorders.

ACQUIRED HEMORRHAGIC VASCULAR DISORDERS

Simple Easy Bruising (Purpura Simplex)

Simple easy bruising is a benign disorder, which occurs predominantly in otherwise healthy women. Onset is often during adolescence or early adult life.

The disorder is relatively common. It is characterized by the occurrence of circumscribed bruises, either on minor trauma or without obvious cause ('devil's pinches'). They are most often seen on the legs and trunk. The bruises are occasionally preceded by pain due to the rupture of a small blood vessel. Although abnormalities are not found in tests of blood coagulation and the bleeding time is usually normal, the tourniquet test occasionally gives a weakly positive result. Some investigators found a high incidence of impaired platelet aggregation with adrenaline and some impairment with ADP and connective tissue. They also detected platelet antibodies in a proportion of the patients and suggested that the 'easy bruising' syndrome may include a proportion of patients with abnormal platelet function on an immune basis.

Simple easy bruising is of importance mainly because of its cosmetic significance and because it may give rise to suspicion of a serious blood disorder. It is probably the most common cause of referral of patients for diagnosis and assessment of unexplained skin bruising. Diagnosis is made on the clinical features and by the exclusion of other causes of purpura. A history of aspirin ingestion should be sought, as it may cause similar bruising. There is no effective treatment; in particular, corticosteriods and other hormones are of no benefit and should be avoided. The patient should be reassured and advised to avoid aspirin when possible. The disorder does not cause excessive bleeding at operation.

Senile Purpura (Involutional Purpura)

Senile (involutional) purpura is a form of purpura that occurs commonly in elderly subjects, mainly on the extensor aspect of the forearms and hands. It occurs equally in males and females over the age of 60 years. An incidence of 2% in the seventh decade, increasing progressively to 25% in the tenth decade. The lesions occur on the extensor surface and radial border of the forearm and on the back of the hand, but they do not extend onto the fingers. They do not occur on any other parts of the body, although they are occasionally seen on the face in relation to spectacle frames, either across the bridge of the nose or along the side pieces. The purpuric areas are large (1–4 cm in diameter), irregular, dark purple, and have a clear-cut-margin. The skin in the affected areas is inelastic, thin, smooth, pigmented, and may show nonpigmented scars; hair is scanty or absent. The purpuric lesions last for varying periods, ranging from a few days to many weeks. The tourniquet test is negative. The lesions are commonly due to minor trauma. There is no effective treatment; in particular, corticosteroids are of no value and indeed may aggravate the disorder and retard resolution of the lesions.

Pathogenesis: Biopsy section of the skin in affected areas shows marked atrophy of collagen; this results in the skin being freely moveable over the deeper tissues. The purpuric lesions are easily induced by a shearing strain to the skin, which tears the vessels passing to the skin because of the excessive mobility of the skin on the subcutaneous tissues. Once the vessels are ruptured, abnormal spread of the blood is permitted by the atrophied collagen fibres. The long persistence of the lesion is due to slow resorption of the blood because of impairment of the normal phagocytic response to extravasated blood.

Purpura similar in distribution and type to that of involutional purpura may occur in patients with rheumatoid arthritis. The association may be related to the duration

of rheumatoid disease. Corticosteroid therapy, however, is likely to contribute in some patients.

SYMPTOMATIC VASCULAR (NONTHROMBOCYTOPENIC) PURPURAS

The term symptomatic vascular purpura is used to describe the purpura occurring in association with a number of disorders, in which the essential lesion is damaged capillary endothelium resulting in increased capillary fragility or permeability.

Diagnosis is, usually based on clinical features, especially the presence of the causative disorder. The platelet count is typically normal, although in some disorders an associated thrombocytopenia may occur. The tourniquet test is commonly, but by no means constantly, positive. The bleeding time is usually normal, but is occasionally prolonged. Symptomatic purpura is a common cause of purpura seen in clinical practice.

Infections

Purpura may occur with many infections, especially severe infections, but it occurs more constantly in some. These include typhoid fever, subacute bacterial endocarditis, meningococcal septicemia, gram-negative septicemia, and smallpox. The purpura is generally considered to be due to toxic damage to the capillary endothelium. However, associated thrombocytopenia, sometimes severe, may be present, especially in septicemia. Intravascular coagulation with a resulting hemostatic defect may also occasionally be a contributing factor. Purpura also occurs as an occasional or rare complication of certain other infections; these include scarlet fever, chickenpox, rubella, measles, tuberculosis, and infectious mononucleosis. In these disorders, thrombocytopenia is an occasional finding, although the purpura usually represents an allergic response to the infection, not related to its severity. The purpura may occur either in the acute stage of the infection or during the period of convalescence.

Sometimes, purpura is the first manifestation of an occult infection in which neither fever nor local signs of infection are present. This is particularly so in children, in whom search for infection, e.g. of the renal tract, should always be carried out in any case of unexplained nonthrombocytopenic purpura.

Drugs

Petechiae and ecchymoses are not so common manifestations of drug and chemical toxicity, other types of skin rash, particularly erythematous, urticarial, and morbilliform rashes, being more common. However, a number of drugs have been recorded as causing purpura, occasionally with mucous membrane bleeding; they include chlorothiazide, carbromal, phenacetin, aspirin, salicylates, amidopyrine, phenylbutazone, hydantoin, barbiturates, chloral hydrate, iodides, gold, arsenic, bismuth, mercury, antihistamines, quinine, quinidine, thiouracils, estrogens, insulin, isoniazid, chlorpromazine, and trinitrin. The development of purpura is due to idiosyncrasy of the patient to the drug, and may be a consequence of specific antibodies to vascular components or of the formation of immune complexes with secondary endothelial damage. The purpura usually clears within a few days to a week of stopping the drug, but pigmentation, when associated, may last up to a month or more. The purpura commonly recurs if the drug is readministered. Some of the drugs listed above may also cause thrombocytopenic purpura.

Uremia

A bleeding tendency may occur in uremia and is occasionally the first clinical manifestation; in general bleeding occurs only with marked nitrogen retention. Epistaxis is the most

frequent symptom, but bleeding into the skin, from the gastrointestinal tract and renal tract, may also occur. In the past, the primary defect was thought to be mainly in the capillary endothelium, but recent studies have shown that a number of abnormalities in platelet function are commonly present. Further, the low hematocrit associated with chronic renal failure appears to play a significant role. Thrombocytopenia may contribute in some cases.

Abnormalities in platelet function include defective aggregation to adenosine disphosphate (ADP), collagen, and adrenaline; decreased platelet retention in glass bead columns; reduction in thromboxane A_2 synthesis; and decreased availability of platelet factor III. In some cases, these abnormalities are improved by dialysis. The responsible factor is not urea but guanidinosuccinic acid and phenol acetic acid have both been implicated. Associated increase in prostacyclin production by the vascular endothelium may compound platelet dysfunction and this may be due to a circulating factor.

von Willebrand factor activity and factor VIII have been reported to be abnormal in chronic renal failure, but the results have been inconsistent and, for the most part, functional defects have not been demonstrated. Some researchers have shown a striking relationship between severity of the anemia and degree of hemostatic defect in these patients. The mechanism is still not clear.

Bleeding in uremia is of particular clinical importance in patients in whom renal biopsy or surgery is contemplated. Before these procedures are performed in a patient with raised serum creatinine, skin bleeding time and platelet count should be performed. A prolonged bleeding time is the best clinical correlate, and the following measures should be undertaken if an abnormality is detected. Thrombocytopenia should be corrected by a platelet transfusion. The hematocrit should be increased to greater than 26% by red cell transfusion. Dialysis may also improve the hemostatic effect.

Two somewhat empirical approaches have been reported to shorten transiently the bleeding time. These are infusion of 10 units cryoprecipitate and intravenous infusion of desamino arginine vasopressin (DDAVP). Subsequently, a conjugated estrogen preparation, Premarin (10 mg daily), has shown to reduce the bleeding time in some uremic patients. There was no improvement in platelet aggregation tests, nor increase in plasma von Willebrand factor activity in these patients. These treatment options are successful in some patients and not in others, suggesting a multifactorial defect; in practice, it may be necessary to combine therapy, monitoring the skin bleeding time meanwhile.

Cushing's Disease and Corticosteroid Administration

Ecchymoses may be seen in Cushing's disease, and are sometimes the presenting manifestation; they may be slow to disappear. The administration of adrenocortical steroids may be accompanied by ecchymoses, easy bruising on minor trauma, and sometimes petechiae. These hemorrhagic skin phenomena occur particularly in women about the menopause. The disorder appears to be due to a vascular defect and is associated with atrophy of collagen; the platelet count and the tests of coagulation are normal. The tourniquet test is positive in some cases. Cessation of steroid administration results in disappearance of the hemorrhagic skin manifestations.

Scurvy

Hemorrhage often occurs in scurvy, and is the major feature of adult scurvy. It is primarily due to the increased capillary fragility which results from defective formation of the intercellular substance of the capillary wall. In addition, a defect of platelet function may be a contributing factor. The skin is the most common site of hemorrhage, which occurs

as both petechiae and ecchymoses of varying size. Hemorrhages may occur anywhere in the skin, but are particularly common in the legs and at the site of trauma; petechiae are commonly perifollicular. Hemorrhage into muscle also occurs, resulting in areas of brawny induration and tenderness. Less common manifestations are epistaxis and conjunctival and retinal hemorrhage; in severe cases, hematemesis, melena, hematuria, and cerebral hemorrhage may occur. The tourniquet test is usually, but not invariably, positive. The anemia of scurvy is described elsewhere.

The diagnosis is suggested by a history of inadequate dietary intake and by the other manifestations of scurvy when present; it is confirmed by the rapid relief of symptoms following adequate vitamin C administration, the purpura commencing to fade within 24–48 hours. Estimation of the ascorbic acid content of white cells and the ascorbic acid saturation test may be used to confirm the diagnosis. Other manifestations of scurvy include follicular hyperkeratosis, particularly on the anterior aspects of the thighs and the ulnar border of the forearms, swelling and congestion of the gums, especially at the site of dental caries, and, in children, bone tenderness and swelling of the extremities. However, the absence of these signs does not exclude the diagnosis of scurvy in the patient who presents with skin hemorrhage.

In adults, scurvy is most often seen in elderly people, particularly men, who live alone and eat inadequate meals, and in chronic alcoholics. In children, it is most often seen in infants on artificial feeding which is not supplemented by vitamin C; thus it may occur in all social groups.

Dysproteinemia

Bleeding may be present in certain disorders characterized by an abnormality of the plasma proteins—the dysproteinemias. These are cryoglobulinemia, hyperglobulinemia, macroglobulinemia, and multiple myeloma. The pathogenesis of the bleeding is not completely understood; in some cases, interference with platelet function is the result of coating of the platelet outer membrane by protein. In many cases, the abnormal protein results in coagulation defects. Increased blood viscosity may cause sludging and increased intracapillary pressure. Thus, troublesome bleeding may be controlled, at least in part, by reduction in level of plasma proteins, either by plasmapheresis or specific chemotherapy.

Cryoglobulinemia: Cryoglobulins are abnormal globulins which have the property of precipitating or gelling in the cold. Cryoglobulinemia is of rare occurrence and is nearly always secondary to some underlying disorder, the most common being multiple myeloma or macroglobulinemia; others include malignant lymphomas and leukemia. In rheumatoid arthritis and systemic lupus erythematosus, immune complexes may behave as cryoglobulins. The purpura of cryoglobulinemia occurs after exposure to cold and may be accompanied by Raynaud's phenomenon. There is sometimes associated urticaria and pruritus. In some cases diagnosis is suggested by 'clotting' of the blood in the syringe. In suspected cases, blood should be taken into a prewarmed syringe and allowed to clot in a water bath at 37°C. The separated serum is then cooled to 4°C; serum containing cryogobulin forms a gel to 4°C but liquefies again when heated to 37°C.

Benign purpura hyperglobulinemia: A rare disorder, described by Waldenstrom and characterized clinically by the appearance at irregular intervals of petechiae, which occurs most commonly on the legs, and sometimes follow exertion or an infection. Purpura also tends to occur under area of pressure. These attacks may be preceded by a feeling of tenderness or swelling in the legs. The disorder is seen mainly in women. Pigmentation commonly develops after a number of attacks. There are usually no positive physical

findings other than the purpura and pigmentation, but in some cases there is moderate lymph node enlargement and hepatosplenomegaly, or associated collagen disorder. The tourniquet test is usually strongly positive. A moderate normochromic normocytic anemia is usual, and the ESR is markedly increased. There is a polyclonal increase in serum immunoglobulins, predominanty IgG, and the histology of the lesions shows evidence of vasculitis. While plasmapheresis may be of value in the acute event, there is no satisfactory long-term therapy.

HENOCH–SCHÖNLEIN SYNDROME (ANAPHYLACTOID PURPURA)

Considered to be a hypersensitivity reaction, allied to acute glomerulonephritis and rheumatic fever. The basic disturbance is a widespread acute inflammatory reaction of the capillaries and small arterioles, resulting in increased vascular permeability and thus in exudation and hemorrhage into the tissues. Bacterial hypersensitivity is the most common cause, but occasional cases result from food and drug hypersensitivity. Foods that have caused anaphylactoid purpura include milk, eggs, tomatoes, strawberries, plums, crab, fish, pork, beans, and peaches. Rare cases following insect bites and smallpox vaccination have been recorded. In some cases, there is no obvious cause. The etiology is not established, although antigenic stimulus by bacteria or food may produce an IgA immune complex-mediated disease. IgA and fibrin can be demonstrated in biopsy specimens from the kidney, and IgA and C3 have been demonstrated in blood vessels from both involved and adjacent normal skin.

Clinical Picture

It may occur at all ages, but most cases are seen in childhood and adolescence. Males are affected more often than females. There is commonly a history of an upper respiratory tract infection with a sore throat 1–3 weeks before the onset, and in such cases a group A beta-hemolytic *Streptococcus* may be isolated from the throat and the antistreptolysin O titre may be raised; occasionally, there is an infective focus at some other site, e.g. skin.

There are four main clinical features—a purpuric rash, joint, abdominal, and renal manifestations; these usually occur in combination, but occasionally only one is present. Most cases present with purpura which is followed shortly by joint and abdominal symptoms. However, sometimes joint or abnormal symptoms first bring the disorder to notice, which may result in diagnostic difficulty, especially in the occasional case in which skin lesions do not develop.

Purpuric Rash

The rash is classically of large and often confluent hemorrhagic macules, but smaller petechial purpuric spots also occur. Initially, the lesions may appear as raised urticarial areas, but within hours they alter to the typical purpuric lesions. They occur most commonly on the buttocks, on the backs of the elbows and extensor surfaces of the arms, and on the extensor surface of the lower leg, the ankle, and foot; they are usually bilateral. They may also appear on the face, but the trunk is generally spared. They occur in recurrent crops which progressively fade over about two weeks. Occasionally, frankly hemorrhagic lesions become bullous and may progress to local necrosis.

Abnormal Manifestations

The abnormal manifestations are due to mural vasculitis and the extravasation of sero-sanguinous fluid into the wall of the intestine. Colicky abdominal pain is common; it may

be accompanied by vomiting, diarrhea, and the passage of bright red blood. The pain varies in severity from mild cramps to severe pain simulating an acute abdominal emergency; most often it is central. Rarely, perforation or intussusception occurs.

Joint involvement with polyarthritis is common; occasionally only one joint is affected. The involvement ranges from mild pain without objective findings to painful swelling of the joints with limitation of movement. The swelling is mainly peri-articular, effusion into the joint being unusual; it lasts only a few days, and resolves without damage. The joints most often affected are the knees and ankles, less commonly the wrists, elbows, and hips. It is not uncommon for swelling to recur, either in the same or other joints. A mild pyrexia is sometimes present, but usually lasts only a few days and seldom longer than a week.

Renal Involvement

Hematuria, either macroscopic or microscopic, is common and is frequently accompanied by albuminuria and the presence of casts. Recovery of the renal lesion is usual, but signs not infrequently persist for many months or years; follow-up shows the development of chronic glomerulonephritis in some cases (probably 5–10%). Rarely, an acute rapidly fatal renal insufficiency develops.

Other Manifestations

Localized areas of edema, most commonly seen on the scalp, the dorsum of the hand, and around the eyes, sometimes unilaterally, are relatively common. Pleurisy, pericarditis, and iritis occur occasionally. Cerebral hemorrhage is a rare complication.

Blood Picture

There are no significant abnormalities other than a moderate polymorph leukocytosis and occasionally a mild eosinophilia. The ESR is usually moderately increased, but it may be normal. The platelet count, bleeding time, and coagulation screening tests are normal. The tourniquet test is moderately positive in about 25% of cases. As indicated above, biopsy specimens from kidney or clinically involved skin show IgA, fibrin and C3 deposition.

Course and Prognosis

The immediate prognosis is excellent, except for those rare cases with intestinal perforation, intussusception, acute renal failure, or cerebral hemorrhage. Typically, recurrences of the clinical manifestations occur over a varying period lasting for a week to several months, with an advantage of about one month; occasionally, they recur for many months even a year or longer. In the 5–10% of cases that develop chronic glomerulonephritis, the prognosis is that of the renal lesion.

Treatment

The disease is usually self-limiting, and treatment is therefore mainly symptomatic to control the joint manifestations and abdominal pain. The administration of antihistamines may lessen exudation.

It has been found that corticosteroids are not useful in the management of the skin manifestation, although they improve painful joint involvement or soft tissue swelling and provide uniform relief of scalp edema. It has also been observed that corticosteroids in adequate dosage usually relieve abdominal pain within 24 hours; if this does not occur, a fixed lesion of the bowel should be suspected. Gastrointestinal bleeding may be well controlled by the drug, but the effect is less dramatic than with other symptoms.

In the event of progressive renal involvement, a trial of corticosteroid therapy or even cytotoxic therapy is warranted. In the occasional case due to food or drug allergy, the offending agent, when identified, should obviously be eliminated.

MISCELLANEOUS DISORDERS

Orthostatic Purpura

The term 'orthostatic purpura' is used to describe the occurrence of purpura on the legs after prolonged standing. It is seen most often in persons with varicose veins and in elderly subjects. There is no demonstrable disorder of the hemostatic mechanism, and the purpura is thought to be due to orthostatic pressure resulting from standing. The effect of this high local orthostatic pressure is also seen in persons with generalized purpuric disorder, as the purpura is often most prominent on the legs.

Mechanical Purpura

Mechanical purpura is due to a local increase in intracapillary pressure. It is most often seen about the head and neck as a result of violent coughing, crush injuries to the chest, or epileptiform seizures. It may also occur on the legs as a result of venous obstruction due to thrombosis, compression by tumor, or the wearing of tight garters.

Fat Embolism

Petechial hemorrhages may be seen in the skin and mucous membranes of patients with fat embolism at the time of onset of stupor, and when present in a suspected case are an important aid to diagnosis. They are most often seen in the skin of the upper part of the chest, the shoulders, and the anterior part of the neck, and less commonly in the conjunctivae and soft palate.

Autoerythrocyte Sensitization

Usually occurs in the adult women and often follows an injury. The main clinical feature is the appearance of repeated crops of large painful ecchymoses. The ecchymoses are preceded by the sudden onset of localized sharp pain or a stringing or burning sensation, and a feeling that a lump is present at the affected site. The area then gradually becomes erythematous, and within an hour or so the ecchymosis appears, which seems to spread from the margin of the erythematous area. The ecchymoses are usually tender and painful for at least several days, and they persist for a week or longer. They tend to occur in crops over several weeks or longer, followed by a period of weeks or months with a few or no ecchymoses. They occur on the extremities, especially the legs, on the trunk and rarely, on the face. Other manifestations that sometimes occur are menorrhagia, abdominal pain, headache, gastrointestinal bleeding, epistaxis, hematuria, and syncope.

The pathogenesis is ill understood. The first attack commonly occurs within a few months of an injury or surgical procedure. It is postulated that the patients become sensitive to the red cell stroma of their own extravasated blood. Thus it has been demonstrated that typical lesion can be produced by the intracutaneous injection of the patient's own blood, and in particular by the erythrocyte stroma; a positive reaction to this test, however, is not invariable, and its absence does not exclude the diagnosis. A high incidence of emotional disturbance has been recorded in these patients, and in some cases the onset or exacerbation of the symptoms has been preceded by emotional stress. It has been found that five psychological components were almost always present in their patients, namely hysterical and masochistic character traits, problem in dealing with their own hostility,

and overt symptoms of depression and anxiety. They suggest that the term 'psychogenic purpura' may be more appropriate than autoerythrocyte sensitization. In a proportion of cases the lesions are factitious, but considerable ingenuity is required to prove that this is so.

Blood examination is normal. Treatment is unsatisfactory, but a short course of corticosteroids may give temporary symptomatic relief. Psychotherapy should be directed towards the treatment of the main emotional disturbances.

DNA *autosensitivity* A somewhat similar clinical disorder in which actual and painful ecchymoses are confined to the legs has been described; the lesions can be reproduced by the intradermal injection of a solution of the patient's white cells or a solution of deoxyribonucleic acid (DNA). Treatment with chloroquine causes prompt clinical improvement, but relapse follows cessation of the drug.

Systemic Disorders

Systemic vascular disorders may be accompanied by an increased capillary fragility. With a positive tourniquet test; these include some cases of collagen disease and amyloid. In the latter disease, coagulation factor deficiencies, thought to be due to absorption of the factor to the amyloid protein, may contribute to the tendency to bleed. Factor X and sometimes factor IX are particularly susceptible. Bleeding is also common in polyarteritis nodosa, presumably as a result of the vascular lesions.

Allergy—Rare cases of nonthrombocytopenic purpura have been described due to food allergy and cold allergy.

CONGENITAL HEMORRHAGIC VASCULAR DISORDERS

Hereditary Hemorrhagic Telangiectasia

Transmitted as a simple dominant trait, Osler–Rendu–Weber disease is a synonym for this condition, which is an uncommon disorder and affects both sexes equally. The basic lesion is the presence in the skin and mucous membranes of telangiectases due to multiple dilatation of capillaries and arterioles. The telangiectases are lined by a thin layer of endothelial cells; because of their thinness they bleed easily, and because they contract poorly, the bleeding is often prolonged.

Clinical Picture

The usual sites of lesion are the skin and mucous membranes of the nose and mouth; however, they may also occur in conjunctivae, bronchi, gastrointestinal and renal tracts, and in the vagina. Lesions in the skin are seen mainly on the face, particularly the ears and cheeks, on the hands, especially the tips of the fingers, and on the feet; in the mouth, they occur on the lips, tongue, cheeks and palate. The telangiectatic spots range in size from a pin-point to lesions up to several millimeters in diameter; they vary in color from purple to bright red, and they blanch on pressure. They are usually raised, but may be flat. Spider-like telangiectases may also occur. The lesions tend to become more numerous and larger with advancing age.

Even though the lesion may be present in childhood, bleeding often does not occur until early adult life. It may occur either spontaneously or following mild trauma. Epistaxis is the most common symptom, and is usually the presenting manifestation. It may occur every day, sometimes several times a day, and it lasts from minutes to hours. Bleeding sometimes lessens or ceases during pregnancy. Much less common manifestations are melena, hematemesis, hemoptysis, hematuria, and menorrhagia. Retinal and cerebral hemorrhages have also been reported. Bleeding from the skin is not as severe as from

mucous membranes, and it may be entirely absent. Pulmonary arteriovenous aneurysm is occasionally present, and splenic enlargement associated with aneurysum of the splenic artery has been described. The liver is sometimes palpable, due to hepatic cirrhosis, a recognized association. or occasionally due to telangiectasis of the liver.

Peripheral Smear

Usually the anemia is proportional to the severity of the bleeding. It is commonly the hypochromic microcytic anemia of iron deficiency, because the chronic blood loss leads to exhaution of the body's iron stores; however, with less severe bleeding, it may be normochromic and normocytic. The platelet count and tests of coagulation and fibrinolysis, suggestive of low-grade disseminated intravascular coagulation. The tourniquet test and bleeding time are also usually normal, but rare cases have been described in which the former is positive and the latter prolonged.

Diagnosis

The diagnosis is based upon the finding of repeated hemorrhages from one, or mainly one, site (particularly epistaxis), the presence of typical lesions in the skin and the mouth, and the family history. As lesions are not always very obvious in the skin, the diagnosis may be overlooked if the mouth is not examined. A family history is usual, but occasionally neither parent gives a history of bleeding; nevertheless; careful examination usually reveals the presence of typical lesions. Diagnostic difficulty may occur when bleeding is predominantly gastrointestinal or renal.

Course and Prognosis

Variable severity of disorder is observed; in mild cases, bleeding is slight and is only of nuisance value, while in severe cases it may cause death. The bleeding often becomes more frequent and severe with advancing years. The chronic anemia associated with frequent and persistent bleeding causes varying degrees of invalidism.

Localized Treatment

Bleeding from the nose is usually the most common problem requiring treatment. Short-term measures include digital pressure, nasal packing, and the local application of topical hemostatic agents. The main long-term treatment is the administration of longer doses of estrogen which significantly lessens epistaxis in many, but not all, patients; it acts by causing squamous metaplasia of the nasal mucosa. The usual dose is 0.25 mg/day ethinyl estradiol; this can be increased to 0.5 mg/day at the end of four weeks if the epistaxis is not well controlled. The daily dose is then varied, either up or down, until a level is reached that keeps the patients epistaxis free. In males, testosterone (2.5–5.0 mg daily) is also given to lessen undesirable feminizing effect. Because of the possible side effects the larger doses of long-term estrogen therapy (particularly jaundice), it should be used only in patients with troublesome epistaxis. Furthermore, the question of these side effects must be carefully explained to the patients. In patients with undesirable side effects, a concentrated estrogen cream applied locally may be a successful substitute. Cautery may be of value, but new lesions often develop about the treated site, and bleeding may recur. The operation of septal dermatoplasty, i.e. resection of the mucosa of the anterior part of the nasal septum and its replacement by a skin graft, may result in permanent control of nose bleeds and should be considered in patients with severe refractory bleeding. With severe recurrent epistaxis, which threatens life, ligation of the external carotid artery

or the anterior ethmoidal artery, or both, may be necessary. Intestinal resection may be necessary in patients with severe gastrointestinal bleeding; however, fresh lesion develop and bleeding usually recurs.

General Treatment

Iron therapy is often indicated to take care of chronic iron deficiency anemia which is common. In more severe cases, parenteral iron is preferred as it enables iron stores to be replenished. Transfusion is sometimes required in patients with severe anemia.

Ehlers–Danlos Disease

A very rare familial disorder, it is transmitted as a Mendelian dominant trait. The basic lesion is a developmental abnormality of the mesenchyme which results in increased fragility of the blood vessels of the skin, together with increased elasticity of the skin and hyperextensibility of joints. A defect of platelet interaction with the abnormal collagen in the vessel wall has also been suggested. The hemostatic defect results in the occurrence of large hematomas following slight trauma or excessive torsion of the skin. There is no effective treatment other than avoidance of trauma.

HEMORRHAGIC DISORDERS DUE TO PLATELET ABNORMALITIES

The platelet function and its important role in normal hemostasis has been considered in foregoing pages.

PLATELET PRODUCTION

Mature megakaryocytes are unique cells in the body in that they can increase their nuclear DNA content within the same nucleus, allowing them to increase protein-producing capacity without undergoing mitosis. Platelets share common antigens with bone marrow megakaryocytes, and the ultrastructural features of platelets are clearly demonstrable in maturing megakaryocytes. Each megakaryocyte produces about 1000 platelets. Any increase in platelet destruction is followed by a change to a higher mean nuclear DNA content of bone marrow megakaryocytes with the production of more active and larger platelets. This is probably a basic mechanism of hemostasis, and is probably under hormonal control.

The platelet count is related to red and white cell counts under normal resting conditions, indicating that the basal platelet count is probably fixed by the hormone acting on the pluripotential stem cell in the bone marrow. The proposed hormone 'thrombopoietin' has been characterized. However, such a factor exists, since thrombocytopenic serum can cause an increase in protein synthesis in megakaryocytes. The control mechanism of platelet production is likely to be complex and to involve more than one chemical messenger.

The formation of platelet from megakaryocyte cytoplasm is also poorly understood. Budding of cytoplasmic processes from the surface of the megakaryocyte within the bone marrow has been accepted, but the evidence for such a mechanism is subjective. Two alternative processes seek to explain the unique platelet volume distribution, which is log Gaussian as opposed to the Gaussian distribution for all other cells. It should be explained that platelet formation without mitosis is a unique process.

The first proposal is that platelets are preformed within the megakaryocyte by the demarcation membrane system, a series of membranes extending throughout the cytoplasm over the mature megakaryocyte. The second theory proposes that megakaryocyte cytoplasm undergoes sequential binary division, an idea supported by a computer model for such

a mechanism. Platelets might be formed and released by either mechanical without the bone marrow or within the lungs, or at both sites. There is no doubt that megakaryocytes leave the born marrow and circulate. Their relatively large size would prevent passage through the pulmonary capillaries. Megakaryocyte nuclei in the pulmonary capillaries, naked megakaryocytes nuclei on the life of the circulatory but not on the right, all suggest that these circulating megakaryocytes lose their cytoplasm in the lungs, giving rise to platelets. The proponents of sequential binary division of megakaryocytes suggest that this might be achieved by physical fragmentation of the cytoplasm within the pulmonary capillary network.

As platelets are involved in both thrombotic and bleeding disorders, abnormalities of platelet production might lead to either dysfunction. Resolution of our knowledge concerning the site and mechanism of platelet production is a prerequisite for a full understanding of platelet disorders.

The life span of platelet once they enter the circulation is about 8–10 days. About 10 percent of the population of platelet in the blood is destroyed each day. It seems probable that there is a small random loss of platelets each day due to the use of platelets in normal maintenance of hemostasis. Measurement of platelet life span is best done by labeling the platelet with radioactive chromium (^{51}Cr) or indium (^{111}In), *ex vivo,* and returning them to the circulation. The disappearance of radioactivity from the blood on succeeding days provides a measure of the platelet survival.

Platelet Antigens and Antibodies

Platelets contain specific antigens, and thus platelet antibodies of several types may occur in the plasma. These antibodies are of significance in platelet transfusion and in the pathogenesis of some cases of thrombocytopenia, especially idiopathic (or immune), neonatal, and drug-induced. Platelet antibodies can be classified as follows:

Alloantibodies

Allo-antibodies (usually anti-P1A1 or anti-Baka (Leka) induced by transfusion and pregnancy. P1A1 antigen is probably located on glycoprotein IIIa, and Baka on the glycoprotein IIb subunit. Platelets also contain histocompatibility antigens, which may induce antibody formation.

Autoantibodies

Autoantibodies, in idiopathic or immune thrombocytopenia, and in certain symptomatic thrombocytopenias. It is interesting to note that antibodies to glycoprotein IIb–IIIa are detected in a proportion of patients with chronic ITP.

Normal Values

The normal values for platelet numbers in peripheral blood vary with the method used for their estimation. The platelet count under phase-contrast is still the preferred visual method. The normal range in health is approximately 150–400 $\times$ 10^9/L , average values being about 250 $\times$ 10^9/L. Platelet counts tend to be subject to error, both because clumping of platelet occurs (particularly with some individuals whose platelets clump in the presence of EDTA anticoagulant used for routine blood specimens) and also because small extraneous particles in the preparation may be mistaken for platelets. However, a careful and experienced worker can produce result that are sufficiently accurate for clinical purposes. The electronic particle counters now widely employed give accurate results, provided that they are calibrated and regularly checked.

The mean platelet volume (MPV) is about 9 femtoliters (fL), and the 'plateletcrit' (PCT) about 0.15–0.3%. These measurements are frequently included in automated systems. Both their accuracy and their clinical value are questionable, although the platelet volume is usually greater in immune than in the other causes of thrombocytopenia.

Physiological Variation

There are no sex differences in counts, and the count in an individual patient tends to remain relatively constant. However, in some normal subjects there is a platelet cycle, with periods of oscillation of 21–35 days. A fall in platelet count may occur in normal women about the time of menstruation. There may also be racial differences, and Mediterranean migrants in Australia have been reported to have significantly lower platelet counts than their Northern European counterparts. The platelet volume has been found to be more in the Mediterranean subjects, suggesting that the circulating platelet mass is the critical parameter in homeostasis.

THROMBOCYTOPENIA

Thrombocytopenia is defined as a reduction in the peripheral blood platelet count below the lower normal limit of 150×10^9/L. Because platelet counts are prone to error, a single platelet count that is lower than normal should always be confirmed by a second count. Further, the thrombocytopenia should also be confirmed by inspection of blood film.

GENERAL CONSIDERATIONS

Platelet Count vs. Bleeding

Hemorrhage is common in thrombocytopenia; nevertheless, many patients with mild to moderate thrombocytopenia, and some with severe thrombocytopenia, go for months or even years without spontaneous bleeding. There is no absolute relationship between the platelet count and the occurrence and severity of bleeding. However, certain broad generalizations can be made. Bleeding is common when the count is less than $30–40 \times 10^9$/L, but is by no means invariable; with counts of less than 10×10^9/L bleeding is usual and is often severe. With values of $40–80 \times 10^9$/L, bleeding is usually absent, although it occurs occasionally. The skin bleeding time, however, has been shown to have a close relationship to platelet count when platelet function is unimpaired.

The conditions under which thrombocytopenia has developed have an important influence on the occurrence of bleeding. When there is associated infection, vascular disease, or metabolic disorder such as the uraemic state, bleeding may occur with relatively mild thrombocytopenia, functional defects contributing to bleeding.

Thrombocytopenia is accompanied by a positive tourniquet test and a prolonged bleeding time.

Positive Tourniquet Test

There is an incompletely understood relationship between the number of platelets and capillary integrity; thrombocytopenia is accompanied by an increase capillary fragility. This is most conveniently demonstrated by the tourniquet test (capillary resistance test of Hess), which, although crude, is a useful part of the examination of any patient with a bleeding tendency.

The tourniquet test is performed by placing a sphygmomanometer cuff around the upper arm and raising the pressure to 100 mm Hg for 5–7 minutes. If systolic blood pressure is

less than 100 mm Hg, the pressure is raised to half way between the systolic and diastolic pressure. Two to three minutes after the cuff has been deflated and the congestion has disappeared, the number of petechiae in an area with a 3 cm diameter, 1 cm below the cubital fossa, is counted. In most normal subjects, the number of petechiae is up to 10, although up to 20 may be present. More than 20 is abnormal. In severe thrombocytopenia, the count is increased up to 100 or more. The petechiae vary in size from pinpoint to pin-head or larger. The tourniquet test is positive in most cases of reasonably significant thrombocytopenia, but is occasionally negative in patients with widespread purpura, the test is redundant and inappropriate.

Excessive Bleeding Time

The bleeding time is the time required for the cessation of hemorrhage from a small puncture wound of the skin made under standard conditions. There are two techniques commonly used, the template method and Ivy's method. In both, a standardized incision is made on the volar aspect of forearm, while the venous and capillary tension is raised by a sphygmomanometer cuff around the upper arm inflation to 40 mm Hg throughout. Variation in the methods related to the cutting instrument which may consist of a scalpel blade, with guard, protruding through a slit in a template or, simply, a disposable lancet. The bleeding time does correlate to the circulating platelet count when platelet function is normal particularly when there is impaired platelet production. An inappropriately short bleeding time may be encountered if there is peripheral platelet destruction because of larger and more reactive platelet produced by 'stressed' bone marrow. Conversely, an unexpected, prolonged bleeding time suggests platelet dysfunction either due to an intrinsic platelet defect or to plasma protein abnormalities, e.g. von Willebrand's disease, or to the presence of certain types of paraproteins. It is essential that drugs affecting platelet function are omitted for an appropriate period (ideally 6–7 days) before bleeding time estimation, and this applies especially to aspirin.

Etiology

Thrombocytopenia may result from impaired platelet production, accelerated platelet destruction, or dilution and or/splenic sequestration (Table 16.2).

Thrombocytopenia is associated with a number of disorders, and other causes should be excluded before assuming a diagnosis of immune (idiopathic) thrombocytopenia.

Table 16.2: Classification of thrombocytopenia

Acquired

More common causes (especially of moderate to severe thrombocytopenia)

- Idiopathic (immune) thrombocytopenic purpura
  - Acute
  - Chronic
- Drugs and chemicals
- Leukemias
- Aplastic anemia
- Bone marrow infiltration—secondary carcinoma, multiple myeloma, malignant lymphomas, myelofibrosis
- Hypersplenism
- Disseminated lupus erythematosus

Contd.

Table 16.2: Classification of thrombocytopenia

Less common causes
- Infection including HIV
- Megaloblastic macrocytic anemia
- Liver disease
- Alcoholism
- Massive blood transfusion
- Disseminated intravascular coagulation

Rare causes
- Thrombotic thrombocytopenic purpura
- Postpartum thrombocytopenia
- Hemangiomas
- Food allergy
- Idiopathic cryoglobulinemia
- Neonatal and congenital

IDIOPATHIC THROMBOCYTOPENIC PURPURA

Primary or essential thrombocytopenic purpura, purpura hemorrhagica, Werlhof's disease, and autoimmune thrombocytopenia are synonyms for idiopathic (immune) thrombocytopenic purpura. This is a disorder characterized by thrombocytopenia in almost all cases due to antibody formation. It is not hereditary or familial, although in occasional cases there is a family history of easy bruising or even of frank bleeding such as expistaxis.

Clinical Picture

It may occur at any age, but is most common in children and young adults. Until the age of about 12 years, the sex incidence is approximately equal, but thereafter females are affected 3–4 times more commonly than males.

Broadly, two clinical types are recognized—an acute self-limiting type, and a chronic type characterized by chronic recurring bleeding over months or years. Although the majority of cases fit into one or other of these groups, there is considerable clinical overlap between them.

Type and Site of Bleeding

The bleeding, as with all bleeding due to thrombocytopenia, commonly occurs spontaneously. It also occurs following trauma, surgery, and dental procedures. Bleeding from wounds tends to occur at once, cease within 48 hours, and does not recur. The skin is the most common site of hemorrhage, and in mild cases it may be the only site. The hemorrhage may take the form of multiple petechiae or ecchymoses, or both. Although petechiae are usually present, ecchymoses may occur in their absence. The petechial spots vary from the size of a pinpoint to a pin-head or somewhat larger; they are not raised and do not blanch on pressure. When fresh, they are red in color, but with time they pass through the color changes of absorbing blood. They characteristically occur in groups or crops, and although they may occur in any part of the body, they are seen especially on the arms and legs, the neck, and the upper part of the chest. They may vary in number from a few scattered crops to innumerable spots covering almost the whole of the body. Ecchymoses vary in size and are initially purple; occasionally, larger hematomas form in the subcutaneous tissue. Hemorrhages are not accompanied by urticaria or erythema.

Bleeding from the mucous membranes is common, although less than skin bleeding; occasionally, it occurs in the absence of skin bleeding. Epistaxis and bleeding from the gums are the most common forms of hemorrhage, but hematuria, menorrhagia and metrorrhagia, and melena are not infrequent. Petechiae similar to those of the skin may be seen in the mouth and nose. Less commonly, hematemesis or hemoptysis occurs. Rarely, there is hemorrhage into the peritoneal or pleural cavities.

Bleeding into internal organs is relatively uncommon, but may be serious. The most important site is the nervous system, especially the brain. Cerebral hemorrhage is the most common cause of death in severe thrombocytopenia. Hemorrhage into the spinal cord and into the meninges may also occur. Rarely, hemorrhage occurs in the tongue, larynx, muscles, fallopian tubes, or ear. Bleeding into joints is very rare.

On examination, the outstanding feature is the absence of physical findings other than those due to the hemorrhage and, when blood loss is severe, to anemia. Subconjunctival and retinal hemorrhages are relatively common. The spleen is enlarged in less than 10% of the cases, and when enlarged is only slightly so. The lymph nodes and liver are not palpable, and there is no sternal tenderness. Jaundice is absent except when there is extensive tissue hemorrhage, e.g. large hematomas, which results in the absorption of large amounts of bile pigment from the broken-down blood. Fever is usually absent, but there may be a moderate rise in temperature with extensive hemorrhage into the tissues or gastrointestinal tract. Rarely, there is chronic ulceration of the legs.

Course of the Bleeding

There are two clinical types, namely acute and chronic, but not uncommonly these overlap.

The acute variety occurs most commonly in children aged from 2 to 6 years, and account for most cases seen in children. It is characterized by a relatively acute onset with hemorrhage into the skin or mucous membranes, or both; the hemorrhage is often severe. Epistaxis is particularly common. There is frequently a history of a viral infection in the preceding several weeks before the onset. In most cases, bleeding ceases spontaneously after a period varying from a few days to 12 weeks; in the remainder it usually ceases within 6 months, but in about 10% of cases it persists and the disorder runs the course of chronic idiopathic thrombocytopenia. In general, bleeding is most severe at the onset, and tends to lessen in severity as time passes. Death is uncommon (<1%); it occurs usually within the first four weeks of onset.

Chronic idiopathic thrombocytopenic purpura is most commonly a disease of young to middle-aged females (females being three times more commonly than males). The onset is usually less abrupt. The severity of the symptoms varies; in some cases it is mild and there may be only recurrent crops of petechiae or 'easy' bruising, while in other cases there may be relatively severe bleeding from mucous membranes, sometimes localized to one site. Occasionally, the first manifestation is menorrhagia occurring at the menarche. In the chronic disease, symptoms are often intermittent, with remissions lasting weeks, months, or even years. In other cases, symptoms persist but fluctuate in severity.

Blood Picture

The main feature is the reduction in platelet count; it occurs in all degrees, values ranging from just below normal to less than 10×10^9/L, lower counts tending to be associated with the acute disease. The platelets sometimes appear to be morphologically abnormal, with large, small and atypical forms. The usual associations of thrombocytopenia—a prolonged bleeding time and a positive tourniquet test—are present. The bleeding time is prolonged

up to 30 minutes or longer. Coagulation studies are normal. Anemia proportional to the degree of blood loss may be present when bleeding is severe; in the early stages, it is normocytic and normochromic, but with prolonged bleeding (e.g. menorrhagia) the iron stores are diminished and the hypochromic microcytic anemia of iron deficiency develops. Rarely, there is an associated autoimmune hemolytic anemia with spherocytosis and other typical features: This is termed Evans' syndrome. The leukocyte count is normal or moderately increased during bleeding episodes. The ESR is usually normal. About 30% of cases have positive anticardiolipin antibodies in the serum. The test is by no means specific, and these antibodies are also commonly present in SLE.

Bone Marrow

Megakaryocytes and their precursors are present in normal, and often in increased, numbers. There is an increase in the percentage of immature cells; these cells have a lesser degree of cytoplasmic granularity. Vacuolization may be present in some cells. In a few cases, there is a moderate increase in mature lymphocytes or in eosinophils. Otherwise, the marrow is normal. When hemorrhage is severe enough to cause anemia there is an associated erythroid hyperplasia, and iron stores may be absent if bleeding has been prolonged.

Diagnosis

Idiopathic thrombocytopenic purpura is characterized by thrombocytopenia with a normal or increased number of megakaryocytes in the bone marrow; the white cell count is normal or slightly increased, and anemia (when present) is proportional to the amount of blood loss. There are usually no positive physical findings other than those due to thrombocytopenia and anemia. As the spleen is enlarged in less than 10% of cases, the presence of splenomegaly in a patient with thrombocytopenia suggests another cause.

The laboratory demonstration of antiplatelet antibodies is a potentially useful diagnostic tool. Recently introduced tests for detecting platelet associated immunoglobulin are standard procedures. Unfortunately, in other conditions frequently associated with thrombocytopenia, e.g. acute leukemia and septicemia, there is increased platelet associated immunoglobulin with considerable overlap with ITP. In spite of these problems of specificity, the tests are clearly superior to the previously available rather insensitive techniques which used indirect measures such as platelet 'injury' to detect antibodies.

EXCLUSION OF OTHER CAUSES

Most of the cases of thrombocytopenia seen in clinical practice are secondary to other disorders, and thus the diagnosis of idiopathic thrombocytopenia can be made only after careful clinical and hematological investigations.

The presence of lymph node enlargement, marked splenomegaly, bone tenderness, fever, anemia out of proportion to the degree of bleeding, or a markedly increased sedimentation rate suggests that the thrombocytopenia is not idiopathic. However, it is not uncommon for none of the above features to be present in other disorders, and their absence does not exclude secondary thrombocytopenia. Bone marrow aspiration is always essential, to exclude leukemia, aplasia, and marrow infiltration, and to demonstrate the typical features of idiopathic thrombocytopenia.

Table 16.3 enlists the main points in differential diagnosis of the disorders most likely to be confused with idiopathic thrombocytopenia. Drug-induced thrombocytopenia is particularly important as it gives a clinical and hematological picture indistinguishable

Table 16.3: Differential diagnosis of thrombocytopenia

	Drug-induced thrombocytopenia	*Acute idiopathic thrombocytopenia*	*Chronic idiopathic thrombocytopenia*	*Acute and subacute leukemia*	*Aplastic anemia*	*Systemic lupus erythematosus*	*Hypersplenism*	*Neoplastic bone marrow infiltration*
History	Present or recent drug ingestion	Commonly recent infection			Sometimes history of drug ingestion or exposure to toxic agents–chemicals, radiation. Exposure sometimes weeks or months and rarely years previously	Symptoms of lupus sometimes present but often absent at onset	Symptoms of disorders causing hypersplenism	Bone pain not uncommon
Clinical features								
Age	All ages	Commonly children	All ages, but most common in women of child-bearing age	All ages		Commonly women of child-bearing age	All ages but usually adults	Usually adults
Splenomegaly	Absent	Uncommon	Uncommon	Usual, but may be absent at onset	Uncommon	Not uncommon	Almost invariable	Usual in myelofibrosis and malignant lymphomas
Lymph node enlargement	Absent	Absent	Absent	Common in some types	Absent	Not common	Common in some disorders causing hypersplenism	Usual in malignant lymphomas
Sternal tenderness	Absent	Absent	Absent	Relatively common	Rare	Absent	Absent	Common
Blood anemia	When present, proportional to bleeding	When present, proportional to bleeding	When present, proportional to bleeding	Anemia out of proportion to bleeding	Anemia out of proportion to bleeding	Anemia often out of proportion to bleeding to	Anemia often out of proportion to bleeding	Anemia usually out of proportion to bleeding

Contd.

Table 16.3: Differential diagnosis of thrombocytopenia

	Drug-induced thrombocytopenia	*Acute idiopathic thrombocytopenia*	*Chronic idiopathic thrombocytopenia*	*Acute and subacute leukemia*	*Aplastic anemia*	*Systemic lupus erythematosus*	*Hypersplenism*	*Neoplastic bone marrow infiltration*
Red cell morphology	Normal	Normal	Normal	Anisocytosis and poikilocytosis usual	Anisocytosis and poikilocytosis uncommon. Sometimes macrocytosis	Normal	Normal	Anisocytosis and poikilocytosis usual and often marked. Nucleated red cells common.
White cell count	Normal or slightly increased	Normal or slightly increased	Normal or slightly increased	Increased, normal or reduced 'blast' cells usual	Reduced	Usually normal or reduced, but may be increased	Usually reduced	Normal, increased or reduced. Myelocytes commonly present
Bone marrow	Megakaryocytes normal or increased in most cases	Megakaryocytes normal or increased	Megakaryocytes normal or increased	Leukemic infilration. Megakaryocytes reduced	Usually hypocellular Megakaryocytes reduced	Megakaryocytes normal or increased	Often hypercellular Megakaryo-cytes normal or increased	Megakaryocytes reduced. Evidence of infiltrating disorder, e.g. carcinoma, myeloma

from that of idiopathic thrombocytopenia; thus a careful history about drug ingestion or exposure to chemical agents must always be taken. Appropriate tests such as those for antinuclear factor, anti-DNA antibodies, and the LE cell test should be performed, as thrombocytopenia may be the first manifestation of disseminated lupus erythematosus. In the appropriate setting, testing for antibodies to human immunodeficiency virus should be carried out.

In cases that come to splenectomy, the spleen should always be examined histologically, as occasionally it gives the first evidence of an unsuspected causative disorder, e.g. lupus erythematosus, tuberculosis, or sarcoidosis.

Bleeding localized to one site. Occasionally, bleeding occurs wholly or mainly from one organ, e.g. in menorrhagia, hematuria, or epistaxis. In such cases, a local cause for the abnormal bleeding may be suspected; this is especially so in patients presenting with menorrhagia.

Pathogenesis

It is thought that idiopathic thrombocytopenic purpura is virtually always due to antiplatelet antibodies. IgG antibodies may be identified in the majority of cases, and bound complement in a proportion. These antibodies are often not demonstrable by the standard serological techniques of agglutination and complement fixation, and other evidence for their existence has been sought.

1. They are often shown by tests that reflect platelet damage induced by antibodies, such as platelet factor 3 availability on platelet serotonin release. However, detection of platelet-associated immunoglobulin or 'bindable' immunoglobulin in the serum is the most useful approach, with greater sensitivity.
2. The transfusion of plasma from a patient with idiopathic thrombocytopenic purpura has been shown to cause thrombocytopenia with clinical purpura in recipients.
3. A transient thrombocytopenia may occur in newborn infants born to mothers with idiopathic thrombocytopenic purpura, suggesting the transplacental passage of an antiplatelet factor from the mother to the fetus.
4. It has been observed that normal platelets can adsorb the factor from the plasma of patients with the disorder; furthermore, the factor reacts with autologous as well as homologous platelets, and is species specific. Even in remission with normal platelet counts, persisting abnormalities in tests of platelet function may result from the subclinical effects of antibodies.
5. Platelets with bound antibody are removed by the reticuloendothelial system in particular the spleen, but with the tendency that increasing platelet antibody load increases hepatic sequestration. Little intravascular platelet lysis occurs. The mechanism of acute idiopahtic thrombocytopenia is less well understood. In about 75% of cases, there is a preceding infection and antibody formation resulting from some reaction between virus and platelets. It may be that the thrombocytopenia represents a hypersensitivity reaction to the infection.

TREATMENT

Patients should be hospitalized for diagnosis and initiating treatment if there is a significant bleeding tendency. Trauma and agents likely to interfere with platelet function, e.g. aspirin, alcohol, must be forbidden. Cough and constipation should be treated to lessen sudden elevations in intracranial pressure.

The main therapeutic measures are the administration of corticosteroids and splenectomy. Immunosuppressive therapy and intravenous administration of immunoglobulin are indicated in selected cases.

The age of the patient is an important factor in determining the therapeutic approach. Thus, although it is not possible to predict from either the clinical or hematological features whether a particular case will be acute or chronic, it is known that most cases in children run an acute self-limiting course, while most cases in adults run a chronic course with recurring attacks. Splenectomy is therefore only rarely necessary in children, while it is commonly performed in adults.

Children

In general, the trend is conservative. In mild cases with bleeding only into the skin or slight bleeding from the mucous membranes, no active treatment may be necessary, although the patient must be closely observed. With more severe bleeding, a course of corticosteroid is given as outlined below. This is usually followed by either a clinical remission or at least sufficient improvement to allow an expectant policy to be followed until the natural remission occurs. If, however, the thrombocytopenia persists for longer than 6 months, the case can be classified as chronic, and splenectomy should be considered if the bleeding tendency is causing more than minor disability. Keep in mind, however, the increased risk of infection after splenectomy, particularly in younger children, and pneumococcal prophylaxis should be instituted. Emergency splenectomy is indicated when despite corticosteriod therapy, the bleeding is sufficiently severe to endanger life; however, with adequate steroid therapy it is seldom necessary. A further option is the intravenous infusion of high dosage immunoglobulin. Because of the comparatively rapid response to this mode of therapy it may be of great value in the emergency situation or when splenectomy is contemplated due to a poor response to corticosteroid.

Adults

The principles are the same as for children, but as the majority of cases tend to run a chronic rather than acute course, splenectomy should be considered earlier, e.g. if after 2–3 months bleeding is troublesome despite corticosteroid therapy. When a chronic case with a long history is seen for the first time, a course of corticosteroids should be tried, but if this fails then splenectomy is indicated.

Corticosteroids

The dose is dependent on the age of the patient and the severity of the bleeding. The initial dose for adult is 1–2 mg/kg prednisolone, higher doses being used if bleeding is severe. The dosage for children is 0.5–1 mg/kg prednisolone. Treatment is usually followed promptly by an improvement in the capillary fragility as shown by the tourniquet test, a shortening of the bleeding time, and a lessening or cessation of hemorrhage; this is followed promptly by an improvement in the capillary fragility as shown by the tourniquet test, a shortening of the bleeding time, and a lessening or cessation of hemorrhage; this is followed by an increase in the platelet count, which is usually obvious within 2–4 days but sometimes does not occur for 10–14 days, and occasionally for 3–4 weeks. In about 25% of cases, there is no improvement. Treatment with full doses is continued for at least two weeks, and for 3–4 weeks if necessary, when the dose is gradually reduced. Cessation of therapy may be followed by a permanent clinical and hematological remission, particularly in children. However, in adults it is more common for a partial or complete relapse to

occur as dosage is reduced, and if an unacceptably high maintenance dosage is required to control thrombocytopenia, splenectomy should be considered. As it is uncommon for such relapsed cases to remit subsequently, there is a strong case for splenectomy to be performed within a few weeks rather than waiting for three months or more, a commonly recommended procedure.

To summarize, the principal aims of treatment with steroids are to protect the patient from the hemorrhagic consequences of severe thrombocytopenia, to reduce the levels of antiplatelet antibodies, and to suppress macrophage function. In chronic cases, the use of steroids is determined by the severity of the bleeding tendency. Prednisolone may be used in the attempt to induce a remission, and a significant proportion of patients respond favorably. Other uses are a postoperative measure in cases of failed splenectomy and in pregnant women after the 5th month of pregnancy.

Splenectomy

Indications

The main indications for splenectomy are chronic cases, particularly in adults, which have not had a sustained response to steroids, and in which troublesome bleeding persists after several weeks. Less common indications are as an emergency measure in both adults and children when, despite adequate steriod therapy, the bleeding is sufficiently severe to endanger life or when cerebral hemorrhage threatens, and in the first 4–5 months of pregnancy, if steroids have not induced a full remission.

Results

Splenectomy results in a sustained clinical remission in approximately 75% of cases. In patients who respond, splenectomy is followed by an improvement in capillary fragility and a shortening of bleeding time, together with lessening or cessation of bleeding, commencing within a matter of minutes, sometimes as soon as the splenic pedicle is clamped. The platelet count starts to rise within a few hours to a few days. The maximum value is reached within three weeks, with an average of about ten days; it usually exceeds normal values, commonly ranging from 5000 to 1000 $\times 10^9$/L. The count slowly returns to normal; occasionally it falls to presplenectomy levels, but the clinical cure persists.

Prediction of Results

At the moment, it is not possible to predict accurately the response of the individual patient to splenectomy. Studies with radioactivity labelled platelet estimating platelet survival time and the site of platelet destruction as indicated by surface scanning are probably not of help in the decision for or against splenectomy There is evidence for a relationship between age and response to corticosteroids and response to splenectomy:

1. Under the age of 45 years, one can expect 80% good responses, but only about 50% in the older age group.
2. Most of the cases that respond to steroids will respond to splenectomy; however, failure to respond to steroids does not imply that splenectomy will be similar ineffective, since about 50% of patients who do not respond to steriods have a good response to splenectomy.
3. A high concentration of platelet-associated antibody is likely to be associated with a poor result.

Failed Splenectomy

About 25% of cases do not respond or do so only temporarily, the platelet count relapsing to presplenectomy values in weeks or months. Rarely, relapse occurs several years later. Occasionally, the count increases slowly over a period of months to years. Continuation or recurrence of troublesome bleeding after splenectomy may be lessened or abolished by steriods or by other forms of therapy. The development of an accessory spleen is reported as a cause of recurrent thrombocytopenia after a successful splenectomy, and should be excluded by splenic scan using heat-treated isotopically labeled red blood cells. However, an exacerbation of the underlying immune disorder is more likely to be responsible.

Mechanism of Response to Splenectomy

The existing evidence suggests that in idiopathic thrombocytopenic purpura platelets sensitized by reaction with plasma antiplatelet antibodies are removed from the circulation by the spleen and the liver. Thus, splenectomy works by removing a major site of platelet destruction; there is also evidence that the concentration of circulating antiplatelet antibodies decreases after splenectomy at least in some patients.

Histology

Histological section of the spleen does not show any characteristic or constant features.

The most common change is an increase in the size of the germinal centres of the lymphoid follicles; other changes observed include an increase in neutrophils and eosinophils in the splenic pulp, and the presence of megakaryocytes in the splenic pulp. Examination of spleen by electron microscopy has shown platelet phagocytosis in splenic macrophages, supporting the belief that this is the major site of removal of antibody-damaged platelets. The importance of careful histological examination of the spleen for evidence of an occult underlying disease in every apparent case of ITP has been emphasized.

Supportive Treatment

Blood transfusion may be necessary when hemorrhage causes severe anemia. Because tranfused platelets are rapidly destroyed by the patient's immune system, platelet transfusion is generally of little value. However, it may be employed in patients with severe bleeding uncontrolled by steroids, when they require splenectomy. The transfusion is probably best commenced just before the first surgical incision is made, a second transfusion being administered after the clamping of the splenic pedicle. Despite its limitation, transfusion may also be used when surgery is needed for bleeding complications or for an unrelated disorder.

Immunosuppressive Treatment

Good results of treatment have been obtained with immunosuppressive agents such as vincristine, azathioprine, or cyclophosphamide. However, the inherent risks of immunosuppression and cytotoxic drugs restrict their use to 'refractory' cases, i.e. those that have failed to respond to corticosteroids and splenectomy, or those in which these methods of treatment are contraindicated. Of the various modalities, vincristine and vinblastine are probably the best choice, with good responses in more than half of postsplenectomy patients so treated. Responses, however, are usually transient, necessitating further courses of treatment. The vincristine is administrated by intravenous push (1 mg/m^2) or by infusion over several hours at a dose of 0.02 mg/kg. Vinblastine has been used at a

dosage of 0.1 mg/kg, and is probably less toxic than vincristine. This may be repeated at weekly intervals for 3–4 doses. If there is no response at this stage, success is not likely.

Immunoglobulin Infusion

IV infusions of immunoglobulin have been shown to result in remissions in both acute and chronic idiopathic thrombocytopenic purpura. The mechanism of action is uncertain, but evidence suggests interference with phagocyte Fc-receptor mediated immune clearance. The therapeutic effect is, however, frequently not transient. In practice, polyvalent intact immunoglobulins are administered in large dosage (0.4 g/kg/day over 5 days) diluted in normal saline. In children, remissions are achieved in almost 100% and are usually permanent. In adults, not surprisingly, the remission rate is less (perhaps 75%), and in the small series so far reported the permanent remission rate is less than half of these. Sometimes, infusions at intervals of weeks can maintain remission.

The unwanted effects seem to be confined to immediate reactions to the immunoglobulin preparation, for example dyspnea, chest pain, nausea, vomiting, and abdominal pain, particularly on the first or second days of therapy. The incidence of these side effects appears to vary with the commercial source of the immunoglobulin preparation (perhaps as a consequence of anticomplementary IgG aggregates), the reported rate varying from 5 to 50%. Viral hepatitis, both hepatitis B and hepatitis C, have been described after use of immunoglobulin fractions, but appear to be rare complications of this therapy.

This form of therapy appears particularly suited to acute situations, prior to surgery or childbirth, and in individuals in whom corticosteroids or splenectomy could be contra-indicated, for example, in cases of thrombocytopenia associated with HIV infection.

Androgens

Androgens have been shown to have a steroid-sparing effect in the treatment of idiopathic thrombocytopenic purpura. The mechanism of this activity is unknown, but possible explanations include displacement of steroids from steroid-binding globulin. Certainly, free antiplatelet antibody has been reported to be reduced by several weeks treatment with danazol, an androgen with reduced virilizing activity.

Pregnancy

The intrauterine death rate with thrombocytopenic mothers varies widely. Neonatal mortality is low, but a significant bleeding defect is present in 10–20%, and thrombocytopenia is 50%.

The management of maternal thrombocytopenia is very much dependent on severity. A platelet count below 50×10^9/L or clinical bleeding is an indication for prednisolone in standard dosage reducing as quickly as possible. In the case of steroid failure, splenectomy may be indicated, ideally in the second trimester. Intravenous high-dose gamma globulin has been used with effect, and should certainly be given to raise the platelet count to cover surgery if required.

The ideal management of delivery is contentious. In the presence of significant maternal thrombocytopenia, attempts should be made to increase the platelet count by either infusion of high-dose gamma globulin, using the 5-day protocol some two weeks before delivery, or by administration of corticosteroids. To lessen the danger to the fetus with potential neonatal thrombocytopenia, several options are available: (a) predelivery attempts to raise the maternal and fetal platelet count either by corticosteroids or infusion of high-dose intravenous gamma globulin started two weeks before delivery; (b) delivery by cesarean

section in the presence of a low maternal platelet count, e.g. below 100×10^9/L; and (c) delivery by cesarean section on the basis of a proven low fetal platelet count performed on a scalp blood sample. In any event, the importance of avoiding a prolonged labour and avoiding forceps delivery would seem self-evident. Any indication that these might occur would weight the decision towards cesarean section.

The infant's platelet count may fall during the first few days after birth, and this should be monitored. In the case of severe thrombocytopenia or hemorrhage intervention with corticosteroids, platelet transfusion or exchange transfusion may be indicated.

IDIOPATHIC CYCLICAL THROMBOCYTOPENIA PURPURA

A rare variant of idiopathic thrombocytopenic purpura in which thrombocytopenia is cyclical, recurring at regular intervals. Most reported cases have occurred in women and have been related to some phase of the menstrual cycle; usually it appears as an exaggeration of the physiological decrease that occurs during menstruation, but cases with the count lowest at the time of ovulation have been described.

SECONDARY THROMBOCYTOPENIA

Most of the cases of thrombocytopenia seen in clinical practice are secondary to some underlying disorder.

Etiology

More Common Causes

Drug-induced Thrombocytopenic Purpura

Careful questioning about the administration of drugs should be carried out in all patients with thrombocytopenia, especially when no definite cause is obvious.

Leukemias

In acute leukemia, thrombocytopenia is almost invariable and is frequently severe. Bleeding is a common presenting manifestation and a frequent cause of death. In chronic lymphocytic leukemia, thrombocytopenia is usual; in the early stages it is usually mild and asymptomatic, but in the later stages it is usually mild and asymptomatic, but in the later stage it may be marked and cause severe bleeding. Efforts should be made to identify the occurrence of autoimmune antiplatelet antibodies if severe thromboctyopenia develops during the course of this disease or malignant lymphoma because specific therapy, as for idiopathic thrombocytopenic purpura, may be successful. In chronic granulocytic leukemia, the platelet count is initially normal or raised, but it falls in the later stages of disease.

Aplastic Anemia

Thrombocytopenia is common in aplastic anemia, especially acute drug-induced cases, and bleeding may be the first manifestation.

Bone Marrow Infiltration

Thrombocytopenia may occur as a consequence of secondary carcinoma, multiple myeloma, myelofibrosis, and the malignant lymphomas. Occasionally, thrombocytopenic bleeding is the first manifestation of secondary carcinoma of bone and multiple meyloma.

Hypersplenism

Hypersplenism is a cause of secondary thrombocytopenia.

Disseminated Lupus Erythematosus

Thrombocytopenia is quite common in this condition. Although the history and clinical examination usually reveal one or more of the other features of the disease, the disorder occasionally presents with thrombocytopenia as the only manifestation.

Less Common Causes

Infection

In acute idiopathic thrombocytopenic purpura of children, there is frequently a history of infection, especially of the upper respiratory tract, several weeks before the onset. In addition, thrombocytopenia occurs as an uncommon or rare complication of certain acute and chronic infections; these include scarlet fever, infectious mononucleosis, measles, rubella (both acquired and congenital), chickenpox, tuberculosis, diphtheria, and subacute bacterial endocarditis. With acute infections, the thrombocytopenia may occur either during the acute phase or during convalescence. The thrombocytopenia probably represents an allergic response to the infection, or a manifestation of bone marrow suppression; there is no relation between the severity of the primary disorder and the occurrence of purpura.

In septicemia, thrombocytopenia is not uncommon in both adults and children. Thrombocytopenia is particularly common with gram-negative bacteremia. *Falciparum* malaria is complicated by thrombocytopenia in severe cases. Immune complex-mediated platelet injury has been suggested as the mechanism for this association.

Megaloblastic Macrocytic Anemia

Megaloblastic macrocytic anemia is commonly accompanied by a mild, symptomless thrombocytopenia; rarely there is a mild bleeding tendency.

Liver Disease

Thrombocytopenia associated with liver disease is most often due to hypersplenism caused by congestive splenomegaly associated with cirrhosis of the liver. However, it occasionally occurs in cirrhotic patients in whom the spleen is not palpable and there is no evidence of portal hypertension. Thrombocytopenia may also occur in severe acute infective hepatitis, probably on the basis of disseminated intravascular coagulation.

Alcoholism

Chronic thrombocytopenia may be seen in chronic alcoholics, in whom it is usually considered to be due to hypersplenism associated with cirrhosis and congestive splenomegaly, or to nutritional megaloblastic anemia. However, an acute transient thrombocytopenia may occur in alcoholics without cirrhosis, related to drinking bouts; the platelet count usually rises within a few days of cessation of alcohol. The thrombocytopenia is due to a direct effect of alcohol intoxication on the developing megakaryocytes.

Disseminated Intravascular Coagulation

Disseminated intravascular coagulation (defibrination) may also be associated with thrombocytopenia.

Massive Blood Transfusion

Thrombocytopenia causing severe bleeding may occur in patients transfused with massive amounts of stored whole blood, when the blood is given either to patients undergoing surgery or to patients with massive bleeding (e.g. gastrointestinal) in whom no surgery has

been performed. The thrombocytopenia is related to the amount of whole blood transfused and the rate of infusion. Thrombocytopenia can be expected when there is replacement of 50% or more of the patient's blood volume. Dilution of the recipient's platelets, the non-viable state of the platelets in stored blood, or their lack in packed red blood cells, are the causes of the thrombocytopenia. Consumption at the site of hemorrhage and activation of coagulation are often additional factors. The platelet count usually returns to normal levels within 3–5 days. Transfusion of 6–8 platelet concentrates is indicated in the presence of hemorrhage when the platelet count falls to levels of around 50×10^9/L in association with massive transfusion.

Unusual Causes

Hemangiomas: Thrombocytopenic purpura has been described in association with congenital hemangiomas in infants; they are usually large and solitary, but are sometimes smaller and multiple. Bleeding often occurs in the first month of life. It is considered to be due to utilization and destruction of platelets in the tumor mass; hypofibrinogenemia and reduction in factors II, V, and VIII occurs in some cases. Successful treatment of the hemangioma usually results in a rise in platelet count and disappearance of the purpura. Treatment with heparin may correct the coagulation abnormalities prior to surgery.

Postpartum Thrombocytopenia

An acute thrombocytopenic purpura occurring about one month after delivery has recently been described; it has also been noted after miscarriage. Most commonly it has occurred in multiparous women; the infants have been normal. The disorder is self-limiting and responds well to corticosteroids, and thus splenectomy is not necessary.

Idiopathic Cryoglobulinemia

Thrombocytopenia may be associated with the rare disorder idiopathic cryoglobulinemia. Response to steroids is generally poor.

Food Allergy

Rare cases of thrombocytopenic purpura have been described in food allergy.

Posttranfusion Thrombocytopenia

A rare syndrome that has been described in middle-aged multiparous women in whom severe purpura develops about 5–7 days after their first blood transfusion, and in whom a platelet iso-antibody is found in the plasma. The antibody is directed against either $P1^{A1}$ or Bak^2 antigens, which these patients lack. The disorder is self-limiting, thrombocytopenia persisting about 3–6 weeks; it is thought to be a result of the development of a platelet iso-anitbody which cross-reacts with the subject's own platelets or which produces immune complexes with the transfused platelets, resulting in an 'innocent bystander' destruction of the patient's platelets. High-dose intravenous immunoglobulin has been found effective, and is probably the treatment of choice. Corticosteroids may also be effective.

Thrombocytopenia with Acquired Immunodeficiency (AIDS)

Thrombocytopenia with acquired immunodeficiency (AIDS) or related syndromes is becoming increasingly recognized. The prevalence of thrombocytopenia is about 10% in patients with persistent generalized lymphadenopathy, and 30% in AIDS. The features are those of immune thrombocytopenia. Corticosteroids may be contraindicated,

depending on the clinical status of the patient, and high-dosage intravenous immunoglobulin may be the best initial treatment. Nevertheless, the response to corticosteroids and splenectomy is similar to that in patients with idiopathic thrombocytopenic purpura.

Treatment

Secondary thrombocytopenic purpura is treated by:

1. Specific measures for relief of the causative disorder.
2. Administration of corticosteriods, which may be of value in some cases when bleeding is troublesome. In general, steroids do not produce a remission as in idiopathic thrombocytopenia, and there is considerable difference of opinion as to a possible nonspecific effect on capillary fragility resulting in a lessening or even temporary cessation of bleeding manifestations.
3. Platelet transfusion in selected cases particularly when the condition is acute and likely to be limited in duration.
4. Splenectomy. In most cases, splenectomy is not indicated. However, it may be indicated in those disorders in which treatment of the underlying disorder is unsuccessful in controlling the bleeding, provided that the immediate prognosis of the underlying disorder is reasonable. Thus, it may be indicated in hypersplenism with thrombocytopenic bleeding, in few selected cases of myelofibrosis, in chronic lymphocytic leukemia, in malignant lymphoma, and in aplastic anemia with refractory thrombocytopenia causing troublesome bleeding.

THROMBOCYTOPENIA DUE TO DRUGS AND CHEMICALS

Thrombocytopenia due to the toxic action of drugs and chemicals on the blood and marrow is not uncommon; most cases occur as a complication of drug therapy, but occasional cases are due to the toxic action of chemicals used in industry or in the home. Drugs that cause thrombocytopenia fall into two groups: (i) those that cause aplastic anemia; and (ii) drugs that cause selective thrombocytopenia, i.e. thrombocytopenia without anemia and neutropenia.

Drugs that Cause Aplastic Anemia

These drugs may cause either selective thrombocytopenia or thrombocytopenia as part of an aplastic anemia. Thrombocytopenia is sometimes the first and only indication of marrow depression. If the drug is stopped as soon as it appears, anemia and neutropenia may not develop; this is especially so with chlorothiazide, organic arsenicals, gold, and sulphonamides. The prognosis is much better when thrombocytopenia is the sole evidence of marrow depression than when pancytopenia develops.

Drugs that Cause Selective Thrombocytopenia

These are all low-risk drugs, purpura occurring only occasionally or rarely (Table 16.4).

Mechanism

The mechanisms responsible for severe drug-induced thrombocytopenia fall broadly into two groups:

i. Those having a direct toxic effect on the bone marrow; and
ii. A hypersensitivity reaction in which the platelets are rapidly destroyed in the peripheral blood, possibly with associated impairment of platelet formation by megakaryocytes.

Table 16.4: Drugs and thrombocytopenia

Drugs that cause aplastic anemia
- Cytotoxic drugs
- Chloramphenicol
- Chlorothiazide
- Tolbutamide, chlorpropamide
- Organic arsenicals

Drugs that cause selective (immune) thrombocytopenia
- Quinidine, quinine, α-methyldopa, digitoxin
- Chlorothiazide, furosemide
- Tolbutamide, chlorpropamide
- Heparin
- Meprobamate, diphenylhydantoin, carbamazepine, valproate
- Sulfonamides, penicillins, cephalosporins, trimethoprim
- Phenylbutazone, salicylates, chloroquine, gold, penicillamine
- Antazoline
- Phenobarbitone, phenothiazines
- Para-aminosalicylic acid, rifampicin

Direct toxic action on the marrow is probably responsible for the thrombocytopenia resulting from most of the drugs. Severe thrombocytopenia occurs only in a small percentage of patients under treatment, and its occurrence is determined by the idiosyncrasy of the patient to the particular drug. However, thrombocytopenia due to ristocetin appears to be due to a direct dose-related nonimmune action of the drug on platelets.

The hypersensitivity reaction with actual destruction of platelets in the peripheral blood is classically seen in quinidine and quinine sensitivity. A single dose of these drugs administered to a person sensitive to them produces a profound fall in platelets, usually in a matter of hours, due to the action of a plasma factor.

It is supposed that the drug acts as a hapten, combines with the platelet, renders it antigenic, and results in the formation of antibody against the drug-platelet complex. On further drug administration, this antibody causes platelet agglutination, and in the presence of complement, lysis. A modification of this theory has been proposed, which suggests that the drug combines with a plasma protein rather than the platelet to form the antigen, which results in antibody formation. When the drug is readminstered, the antibody combines with the antigen (drug plus plasma protein) to form an immune complex which is adsorbed onto the surface of the platelet, resulting in its removal by the reticuloendothelial system. According to this theory, the platelet is involved in the immune reaction as an innocent bystander' on which an extrinsic immune complex reacts. More recent studies suggest that both platelet membrane (glycoprotein Ib and glycoprotein IX) and plasma protein is required for antibody formation and for subsequent platelet damage, and it appears that von Willebrand factor is involved in this complex system.

Clinical Picture

Usually there is a history of administration of the drug for days, weeks, or even months, followed by bleeding in a matter of hours up to several days following the last dose. In cases due to marrow depressing agents, e.g. gold, there is sometimes an interval of weeks or more between the last dose and the onset of bleeding. The bleeding is sometimes mild and limited to the skin, but frequently it is severe with extensive mucous membrane hemorrhage and 'blood blisters' in the mouth, as well as skin petechiae and ecchymoses.

Severe hemorrhage of sudden onset is especially characteristic of those drugs in which hypersensitivity can be demonstrated, especially quinidine, quinine, and digitoxin. With acute severe bleeding, constitutional symptoms are common—chills, headaches, generalized aches, fever, abdominal pain, nausea, vomiting, and itching of the skin. Withdrawal of the drug is followed by cessation of bleeding within a few hours or days. Quinidine purpura is more common in females than males.

Thrombocytopenia due to quinine is not as common as that due to quinidine, but it is of special importance because quinine is present in a number of commonly consumed drinks such as 'tonic' waters and other bitter drinks, and in certain proprietary medicines which may be self-administered. The term 'cocktail purpura' has been used to describe purpura occurring after ingestion of drinks.

A unique drug-induced thrombocytopenic syndrome is associated with heparin administration in 3–5% of patients receiving the drug for more than 5–7 years. In a small proportion of individuals so affected, thrombocytopenia is accompanied by thrombosis, either venous or arterial, often widespread and resulting in severe morbidity and significant mortality. Usually, the patient's plasma contains an antibody which aggregates normal platelets in the presence of 0.5–1.0 U heparin/mL *in vitro*. A more sensitive laboratory test may be release of 14C-labeled serotonin from platelets in a similar *in vitro* system. An early diagnosis is essential, and routine platelet counts should be carried out on patients receiving heparin for more than five days. Treatment consists of suspending heparin and administering dextran intravenously, and usually warfarin. There is hope that recently developed low molecular weight heparin analogues might not cross-react with the patient's antibodies, and will thus represent alternative and more effective therapy for this grave illness.

The characteristic blood picture is that of thrombocytopenia without anemia or neutropenia. There may be a moderate leukocytosis during the bleeding. The bone marrow shows a normal or increased number of megakaryocytes, many of which show absent or reduced granularity.

The diagnosis is suggested by the history of drug ingestion, the severity of the bleeding manifestations, the presence of constitutional symptoms, and spontaneous remission on cessation of the drug. Usual response to a test dose establishes the diagnosis with certainty, but as test doses may produce dangerous bleeding they are unsafe and should be avoided.

Due to the importance of avoiding the offending drug in the future, special tests designed to support the diagnosis of causal relationship between the thrombocytopenia and the suspected drug should be carried out. These tests are commonly positive when the thrombocytopenia is due to quinidine, quinine, digitoxin, and heparin in which antibody-mediated hypersensitivity has been demonstrated to be the mechanism of the thrombocytopenia, but may be positive with other drugs, although less commonly so. The demonstration of immunoglobulin binding to platelets in the presence of the offending drug will probably prove to be the most sensitive laboratory test. Other tests, in probable order of sensitivity, are the complement fixation test, platelet serotonin and platelet factor 3 release test, the clot retraction inhibition test, and platelet agglutination and lysis test. The platelet agglutination and lysis tests are relatively insensitive, and often give negative results when other tests are positive.

A negative result for a specific drug does not exclude it as a cause of the thrombocytopenia.

Infrequently, thrombocytopenic purpura first develops in the postoperative period; in such cases it may be due to sedatives, analgesics, or antibiotics, and infection should be excluded.

Prognosis

In selective thrombocytopenia, the platelet count returns to normal and the bleeding ceases within a few hours to a few days after stopping the drug; recovery is nearly always complete within 7–14 days. Occasionally, death occurs, usually from cerebral hemorrhage. Heparin-induced thrombocytopenia with thrombosis carries a worse prognosis. When thrombocytopenia occurs as part of an aplastic anemia, the prognosis is that of aplastic anemia.

Treatment

Treatment consists of:

a. Immediate cessation of the offending drug. Once a patient has developed thrombocytopenia due to a particular drug they should never be given that drug or chemically related drugs again, and should carry a warning card to show to future medical attendants.
b. The administration of corticosteroids in patients with severe bleeding, as for acute idiopathic thrombocytopenic purpura, and
c. Blood transfusion to replace blood loss. It is probable that in cases caused by antibodies, transfused platelets are rapidly destroyed. Nevertheless, if bleeding appears to be life-threatening, platelet transfusion should be given. Splenectomy is without beneficial effect and is contraindicated.

THROMBOTIC THROMBOCYTOPENIC PURPURA

Thrombotic microangiopathic hemolytic anemia and thrombohemolytic thrombocytopenic purpura, are synonyms for thrombotic thrombocytopenic purpura, a rare disorder characterized by the occurrence of fever, thrombocytopenic purpura, hemolytic anemia, fluctuating neurological disturbances of variable nature, and renal disease. Any one of these features may be absent, and often all five are present only in the terminal stages. Abdominal pain and jaundice are not common.

The disorder is of rapid onset, purpura or rapidly developing anemia usually being the first manifestations. It may occur at any age, but is most common in young adults. The spleen may be palpable. Blood examination shows a severe hemolytic anemia, thrombocytopenia, and leukocytosis, sometimes with a leukemoid reaction; the morphological features of microangiopathic hemolytic anemia are typically present. The direct Coombs' test is usually negative. Bone marrow examination reveals erythroid and often myeloid hyperplasia, and a normal or slightly increased number of megakaryocytes; sections of aspirated marrow show diagnostic hyaline thrombi in a significant proportion. A polyclonal increase in immunoglobulins may be found. Laboratory evidence for disseminated intravascular coagulation is usually minimal or absent.

The etiology is unfamiliar; it appears to be related to the collagen diseases, and it is possible that hypersensitivity plays an etiological role. Associations are recent infections, pregnancy, oral contraceptives, and a genetic susceptibility. At postmortem, multiple hemorrhages, usually petechial, are found macroscopically, while microscopically the characteristic feature is the presence in many organs of hyaline thrombi in the capillaries and terminal arterioles. The clinical manifestations are due to widespread intravascular platelet thrombi. The pathogenesis is not known, but evidence points to a vascular endothelial cell abnormality. Defective prostacyclin release, impaired fibrinolysis, and abnormal von Willebrand factor multimers in plasma, all related to endothelial functions, have been reported in this disease. A platelet agglutinating factor has also been described. The administration of plasma or cryoprecipitate can reverse some of the abnormalities.

Diagnosis can sometimes be established by demonstration of the typical thrombi in a biopsy specimen, for example from the gingiva. There is no authoritative treatment. A number of empirical measures, including corticosteroids, splenectomy, and anticoagulants, appear ineffective in most instances. Recent experience confirms that plasmapheresis (and in some cases repeated plasma infusions) is of unequivocal benefit in most cases. Because of variable responses a combination of daily plasmapheresis, antiplatelet drugs, and corticosteroid in high dosage should be instituted on diagnosis. In earlier reports, the disease was almost uniformly fatal, usually within a matter of weeks. A growing number of cases (up to 75%) surviving long periods have been recorded more recently, and clearly survival has been influenced by treatment.

NEONATAL AND INHERITED THROMBOCYTOPENIAS

The platelet count of full-term newborns is only slightly lower than that of older children and adults. Premature infants, however, have lower counts, and this should be kept in mind during the investigation of bleeding in a premature infant. Neonatal thrombocytopenia may be acquired or inherited, and presents at birth or within a few hours of birth. However, inherited disorders may not present clinically for several months or longer after birth; they may remain a problem throughout life.

It should not be forgotten that petechiae not due to thrombocytopenia are quite commonly seen in normal newborn infants; they are usually confined to the head and upper chest, and disappear in a short time; they are considered to be due to a temporary increase of venous pressure during delivery (Table 16.5).

Table 16.5: Neonatal and congenital thrombocytopenia

- Immune
  - Autoimmune: Mothers with chronic idiopathic thrombocytopenia purpura
  - Isoimmune (alloimmune): Platelet group incompatability

Infections
  - Congenital or neonatal
- Drug administration to mother
- Congenital megakaryocytic hypoplasia
  - Isolated
  - Associated with congenital abnormalities or pancytopenia
- Hereditary
  - Sex-linked: Pure form, Aldrich's syndrome
  - Autosomal: Dominant or recessive
- Congenital leukemia
- Giant hemangioma

IMMUNE THROMBOCYTOPENIA

It may arise in cases in which the mother has suffered from idiopathic thrombocytopenic purpura, and is due to the transplacental passage of the antiplatelet autoantibody from the mother to the fetus. It occurs in approximately 50% of infants born to mothers who are thormbocytopenic at the time of delivery, but is less common when the mother's platelet count is normal. It may occur in infants of both splenectomized and nonsplenectomized mothers. The purpura appears within 24 hours of birth, is usually mild, and spontaneously disappears within several weeks; however, it is sometimes severe and may occasionally result in death.

Immune thrombocytopenia may also occur in infants from mothers who do not have idiopathic thrombocytopenic purpura. The infant possesses a platelet antigen lacking in the mother, usually PL^{A1}. Maternal antibodies directed against PL^{A1} cross the placenta to the fetal circulation, and produce a condition known as isoimmune (alloimmune) neonatal thrombocytopenic purpura; this condition has been reported as the cause of about 20% of cases of immune neonatal thrombocytopenia. In general, bleeding manifestations are more severe in the iso-immune than autoimmune thrombocytopenic purpuras. Thrombocytopenia may also occur in erythroblastosis fetalis; its mechanism is uncertain; but it is probably related to either the hemolysis or to exchange transfusion.

Drug Ingestion by the Mother

Two types of this form of neonatal thrombocytopenia exist. In one, neonatal thrombocytopenia is associated with immune drug-induced thrombocytopenia in the mother, with passage of the antibody across the placenta to act on the infant's platelets. In the second, the mother is not thrombocytopenic; this variety has been described particularly with thiazide drugs, especially when given for prolonged periods, e.g. up to three months, during pregnancy. It appears that the marrow of the affected infants is unduly susceptible to the drug.

Infection

Thrombocytopenia occurring at birth or in the first two days of life may result from almost any form of infection, but is particularly common in infections such as cytomegalic inclusion disease, disseminated herpes simplex infection, congenital toxoplasmosis, and congenital syphilis.

MEGAKARYOCYTIC HYPOPLASIA

Megakaryocytic hypoplasia occurs as an isolated phenomenon in an otherwise healthy child, or in association with a syndrome of congenital abnormalities. The congenital abnormalities most commonly associated with megakaryocytic hypoplasia include bilateral absence of the radii, the rubella syndrome, and pancytopenia with multiple congenital abnormalities (Fanconi's syndrome).

INHERITED THROMBOCYTOPENIAS

A number of genetically distinct forms of inherited thrombocytopenia have been described. These include sex-linked thrombocytopenia, autosomal dominant, and autosomal recessive thrombocytopenia. These conditions produce life-long bleeding disorders of variable severity, and bleeding in the newborn is infrequent. Inherited thrombocytopenia occurring in the pure form may be associated with functionally abnormal platelets.

In three of the inherited disorders, distinctive features are present. Aldrich's syndrome is characterized by eczema, recurrent infections, and thromocytopenia; most infants eventually die, by the age of three years. The May–Hegglin anomaly is familial, and characterized by thrombocytopenia (often mild and usually asymptomatic), with giant platelets and Dohle bodies in the cytoplasm of the granulocytes. The Bernard–Soulier syndrome, with giant platelets and functional defects, is also characterized by a degree of thrombocytopenia in most patients.

PLATELET TRANSFUSION

TYPES OF PLATELET PREPARATION

The transfusion of viable, physiologically active platelets can be achieved:

1. With fresh whole blood
2. With platelet-rich plasma (PRP) or platelet concentrates (PC) obtained from fresh whole blood.
3. With platelet concentrates prepared by plateletpheresis using a continuous or intermittent flow cell separator. Plateletpheresis has the decided advantage that a clinically useful transfusion can be achieved using platelets harvested from a single donor. A comparable infusion of platelets prepared from donor units of 500 mL whole blood necessitates pooling from about six donors (total 5–6 × 10^{11} platelets).
4. With autologous (own) platelets collected by plateletpheresis and stored frozen until required.

In the majority of cases, whatever be the underlying disease, platelet concentrates are used. Associated anemia may be treated by the transfusion of packed red cells. Fresh whole blood is more difficult to obtain and, in practice, is effectively replaced by the combination of packed red cells and platelet concentrates.

The methods of preparation of platelets are undergoing constant revision, but most currently closed system of plastic packs. Meticulous technique in the collection of the blood is of paramount importance in obtaining a satisfactory yield of platelets; in particular, any clotting must be avoided as the presence of even small amounts of thrombin can seriously damage platelets. Preparation is usually carried out at ambient room temperature (22°C) which results in a reasonable life span when reinfused into the patient, and allows longer storage before administration. With the introduction of newer plastics, the container bags allow rapid gas exchange, ensuring maintenance of pH greater than 6.0. The storage period is consequently longer, and platelets may remain viable for transfusion for up to 7 days.

CLINICAL EFFECT

The clinical effect is dependent upon the number and viability of the platelets given, the mechanism of the thrombocytopenia and the presence or otherwise of significant splenomegaly. A poor response may also be due to associated infection, fever, consumption coagulopathy, or the presence of antibodies, either iso or auto.

Platelet viability is determined by the time interval between collection and use, and by the care in preparation. In general, the shorter time between the commencement of withdrawal of the blood from the donor and the completion of transfusion in the recipient, the more effective is the transfusion. Thus, the time should be as short as possible.

The effect is determined by clinical assessment of the bleeding tendency, combined with estimation of the platelet count. A transfusion is regarded as successful when the bleeding manifestation for which it was given is controlled for a period of at least 48 hours. Control of the bleeding tendency commonly outlasts the rise in the platelet count when this occurs (it should be 10–20 × 10^9/L/m^2 one hour post-transfusion for each unit of platelets infused). In some cases there is no significant rise despite clinical improvement in the bleeding.

ISO-(ALLO)IMMUNIZATION AND REACTIONS

Platelets contain isoantigens and till date about twelve have been identified. There are no naturally occurring isoantibodies, and antibodies, when present, they are the result of immunization by previous transfusion. Antibodies are most readily detected by

complement fixation and platelet release techniques. The latter has been shown to be sensitive to isoantibodies and these have HLA or membrane glycoprotein specificities. Tests may be used to select compatible donors for platelet transfusion, but there are specialized and not widely applied at present. Direct cross-matching procedures are also described but not widely practised.

Although immunization from previous platelet administration is clinically less of a problem than first anticipated, platelet isoantibodies are readily formed, and may impair the effectiveness of repeated transfusions.

Places where sensitive techniques are available, isoimmunization can be shown to occur after very few transfusions. For this reason, care should be taken in selecting donors for patients who may require prolonged support as, for example, in aplastic anemia. This is especially the case if bone marrow transplantation is contemplated, when care must be taken to avoid immunization against HLA antigens. It is the basis for the increased use of single-donor platelet preparations from plateletpheresis of close relatives or HLA-identical individuals in such donors. Patients with acute leukemia, on the other hand, who are undergoing treatment that is immunosuppressive seem less liable to develop antibodies, and may be sustained for long periods using platelets from ABO and Rh compatible donors.

Weak reactions with fever and chill are not uncommon, but serious transfusion reactions do not appear to occur; in patients with mild reactions complement-fixing platelet antibodies may be demonstrated in vitro, and there is failure of the transfusion to increase the platelet count. Marked reactions, when they occur, are probably due to the presence of associated leukocyte antibodies acting against transfused leukocytes.

A serious hazard of platelet transfusion is transmission of virus, including human immunodeficiency virus (HIV) and hepatitis, but donor testing for hepatitis B antigen and HIV antibody reduces the risks.

INDICATIONS

Platelet transfusion should be limited to patients with severe thrombocytopenia with specific indications.

A vital factor in determining the effectiveness and thus the indications for platelet transfusion is the mechanism of thrombocytopenia. In cases due to decreased marrow production, the platelets generally survive long enough to be effective, provided they are properly prepared and given in adequate numbers. However, in cases due to excess platelet destruction, they seldom survive sufficiently long to cause clinical improvement. Significant splenomegaly with sequestration and possibly destruction of platelets may also lessen effectiveness.

The indications are both surgical and medical; the surgical indication is often prophylactic.

Medical

In potentially self-limiting thrombocytopenias of short duration, e.g. due to drug reaction (especially when due to marrow depression), chemotherapy, radiation, and massive transfusion, platelet transfusion is indicated when there is serious bleeding.

In thrombocytopenia of longer duration, as in aplastic anemia or bone marrow infiltration, platelet transfusion may be used temporarily to tide the patient over an exacerbation of bleeding. This is especially so when there is an acute additional factor which is depressing the platelet count and increasing clinical bleeding, e.g. infection in aplastic anemia, chemotherapy in acute leukemia, chemotherapy or radiation in lymphomas

and chronic leukemias. There is reasonable evidence that regular prophylactic platelet transfusions to individuals with platelet counts persistently lower than 20×10^9/L will reduce the incidence of spontaneous hemorrhage and probably mortality.

Platelet transfusion is particularly indicated with suspected or proven internal bleeding, e.g. intracranial, thoracic, or peritoneal bleeding.

Surgical

Prophylactic transfusion may be indicated when significant bleeding is expected, as in patients with thrombocytopenia due to marrow insufficiency or depression, e.g. in aplastic anemia, and lymphoma or leukemia. In general, it is not indicated in splenectomy for idiopathic thrombocytopenic purpura, although it may be used in occasional cases. Platelet transfusion is indicated for surgical procedures in patients with significant platelet dysfunction.

FUNCTIONAL PLATELET DISORDERS

A bleeding disorder can result not only from a decrease in platelet number (quantitative defect), but also from an abnormality of function (qualitative defect). Qualitative defects may result in excessive bleeding, even though the platelet count is normal, which is the case in the majority of instances of disordered platelet function.

SPECIAL TESTS

Skin Bleeding Time

The bleeding time is the most useful clinical test to confirm the presence of a suspected defect in platelet function. If the bleeding time is normal, a platelet abnormality is of limited clinical significance. In the presence of thrombocytopenia, prolongation of the bleeding time beyond that expected for the platelet count is suggestive of coexisting platelet dysfunction.

Morphology

Morphology of platelets is of value in the diagnosis of a number of functional disorders, both congenital and acquired. Morphological features include size and shape, and the appearance of granules and other cytoplasmic features. The morphology may be assessed on routine blood smear, by fluorescent microscopy using specific stains or fluorescent antibodies, and by transmission electron microscopy to identify dense bodies, alpha granules, the microtubules, the surface-connected cannalicular system, and other ultrastructural details.

Platelet aggregation tests are cornerstones for defining functional defects in the clinical laboratory. Aggregation is carried out in transparent cuvettes at 37°C using stirred platelet rich plasma. Agonists are added to individual fresh samples, the reagents being chosen to test different pathways of platelet activation. Aggregation causes a decrease in the turbidity of the platelet suspension. This decrease in turbidity (or increase in light transmission) is detected by a photoelectric cell and recorded on moving paper. Numerous modifications have been made, including the automated, simultaneous measurement of adenosine disphosphate and triphosphate release by chemiluminescence, or the recording of aggregation of platelets in whole blood by measuring the changes in electrical impedance. The agonists commonly used are collagen, ADP, arachidonic acid, adrenaline, thrombin and ristocetin.

Platelet Factor 3 Availability Assay (PF3 Availability)

This assay measures the platelet contribution to clotting of blood, which can be substituted by a number of phospholipids. It is based on the shortening of the Russell's viper venom (or kaolin) clotting time due to the presence of platelets. Occasionally, patients have been described who have an isolated defect in PF3 availability but, for the most part, more specific tests of platelet function, in particular platelet aggregation, provide greater specificity, and the assay is not widely performed in routine laboratories.

Platelet Adhesion

Measured by retention of platelets in glass bead columns, has been superseded by more specific tests, especially in the diagnosis of von Willebrand's disease. This technique reflects a combination of adhesion and aggregation of platelets and, perhaps for this reason, is prone to artefact, adhesion in glass bead columns is dependent on many factors, such as the anticoagulant used, the rate of perfusion of blood through the column, the type of tubing, and characteristics of the glass beads. Abnormal adhesion is common in thrombasthenia, Bernard–Soulier syndrome, von Willebrand's disease, myeloproliferative disorders, uremia, and congenital aggregation disorder described below.

Prothrombin Consumption Test

The prothrombin consumption test measures the residual prothrombin remaining in serum after the clotting of whole blood. The test depends on both the coagulation cascade and presence of normal numbers of functional platelet. While useful as a screening test, more likely to define functional abnormalities, and for this reason many routine laboratories would not include it in their normal test profile.

Specialized Techniques and Research Methodologies

There are now more sophisticated techniques for defining the physiology and biochemistry of platelet function in research laboratories. These include: (a) the identification and analysis of membrane receptors by electrophoresis and antibody probes; (b) measurement of secreted proteins, e.g. β-thromboglobulin and platelet factor IV, using specific immuno-assays; (c) analysis of fatty acid metabolies, particularly those of arachidonic acid by radio-immuno-assay or chromatograhic techniques; and (d) the measurement of intracellular calcium concentration changes by fluorescent markers. The application of these and other techniques has led to the better definition of functional defects over the past two decades.

CLASSIFICATION OF PLATELET DYSFUNCTION

Both congenital and acquired disorders exist in which platelet functional defects are clearly defined. They may be classified according to etiology as given in the Table 16.6.

CONGENITAL QUALITATIVE PLATELET DEFECTS

Thrombasthenia (Glanzmann's Disease)

Glanzmann in 1918, described a bleeding disorder associated with a normal platelet count and defective clot reaction. It is a familial disorder, occurring in both sexes and transmitted as an autosomal recessive gene. The bleeding time is long in spite of a normal platelet count and normal platelet morphology. The platelets fail to aggregate in response to ADP, collagen, and thrombin, but do respond to ristocetin. There is decreased or absent glass bead retention and platelet factor 3 availability with a defective release reaction. The defect

Table 16.6: Classification of disorders of platelet function

Congenital
• Membrane receptor defects
• Glycoprotein IIb/IIIa deficiency—Glanzmann's thrombasthenia
• Glycoprotein Ib deficiency—Bernard–Soulier Syndrome
• Pseudo-von Willebrand disease
Enzyme defects
• Phospholipase deficinecy—aspirin-like defect
• Cyclooxygenase deficiency—aspirin-like defect
• Thromboxane synthetase deficiency
Granule defects
• Storage pool deficiency
• Alpha granule deficiency—Gray platelet syndrome
Platelet procoagulant activity defects
• Acquired
• Stem cell disorders—leukemia, myelodysplasia, myeloproliferative
• Drugs
• Dysproteinemias—myeloma, macrogolbulinemia
• Uremia
• Miscellaneous—autoantibodies, disseminated intravascular coagulation (DIC), posttransfusion, etc.

is located in the membrane with a lack of glycoprotein IIb-IIIa complex, which is the point of attachment with fibrinogen, which in turn is required for platelet-platelet binding in the aggregation process. Clinically, the disorder is characterized by a tendency to bruise after even minor trauma, excessive and prolonged bleeding after cuts and abrasion, epistaxis, and menorrhagia which can be a very troublesome problem. Deep hematoma and hemarthroses are rare. The heterozygote is usually unaffected, although occasionally a mild bleeding tendency with easy bruising has been described.

BERNARD–SOULIER SYNDROME

A very rare, autosomal recessive disorder characterized by a severe bleeding tendency. The pattern of bleeding is similar to that of Glanzmann's thrombasthenia, and heterozygotes have normal hemostasis. The syndrome is characterized by a combination of moderate to severe thrombocytopenia, large platelets on peripheral blood smear, and a very prolonged skin bleeding time. The platelets aggregate with physiological agonists (although slowly with thrombin) but fail to 'agglutinate' with ristocetin, a similar pattern to that seen with classical von Willebrand's disease. The defect, however, lies in the deficiency of membrane glycoprotein Ib and associated glycoprotein IX. There may also be an abnormality of the glycoprotein V. The only available treatment of these patients, and indeed all of the congenital function disorder, is the judicious use of platelet transfusion in the face of a bleeding episode or as prophylaxis for surgery. The major problem is then the development of antiplatelet iso-(allo-) antibodies, rendering further transfusions ineffective.

DEFECTS OF PLATELET ENZYMES

Familial deficiency of the enzymes, cyclooxygenase and thromboxane synthetase, involved in the metabolism of arachidonic acid and production of thromboxane A_2 have been reported. These are rare, and their genetic transmission uncertain. The bleeding defect is mild, and it is of clinical significance only in the case of serious trauma or surgery. The bleeding time is prolonged to a mild or moderate degree, and the platelet aggregation

to ADP, collagen, and arachidonic acid is abnormal. Differentiation from storage pool disease is achieved by studying the effect of arachidonic acid and endoperoxide analogs in aggregation, and identifying normal dense bodies and their contents. Platelet transfusion to cover trauma or surgery is usually the only therapy required.

GRANULE DEFECTS

Deficiencies of specific granules may occur independently—dense body deficiency (δ a storage pool disease), Gray platelet syndrome (α granule deficiency)—together (αδ storage pool disease); or as a component of a group of inherited disease with multiple abnormalities.

The group of inherited disorder in which δ storage pool deficiency is one of a number of defects consists of Hermansky–Pudlack syndrome (oculocutaneous albinism, bleeding tendency, and pigmented macrophages in the bone marrow), Wiskott–Aldrich sysdrome (thrombocytopenia, eczema, and immunodeficiency) and Chediak–Higashi syndrome (susceptible to infection, thrombocytopenia, and defective pigmentation of skin and retina). Thrombocytopenia with absent radii is said to be associated with storage pool disease in some cases.

Storage Pool Disease (δ-SPD)

It causes a mild to moderate bleeding tendency similar to that induced by aspirin. Inheritance is autosomal dominant. Platelet aggregation with ADP and collagen is defective, but to arachidonic acid is normal. Absence of the dense bodies can be documented using electron microscopy, fluorescence with mepacrine treated platelet, or by the grossly subnormal secretion of adenine nucleotides.

Gray Platelet Syndrome (δ-SPD)

It is an extremely rare autosomal dominant disorder with a mild bleeding diathesis, epistaxis, easy bruising, and ecchymoses. The characteristics are prolongation of skin bleeding time, mild to moderate thrombocytopenia, and enlarged platelets with an unusual gray color on routine peripheral blood film. The platelets lack the a granules, and are deficient in the constituents of these granules. The defect is almost certainly one of the granule proteins which are unique to megakaryocytes and platelets (e.g. β TG) is increased, suggesting synthesis in the marrow but subsequent leakage in the megakaryocyte or platelet. Marrow fibrosis is seen in this condition, and is presumably a consequence of elevated local concentrations of the platelet-derived growth factor and of other components that normally concentrate in the granule, destined for release at sites of tissue injury far from the site of synthesis.

Combined Defects (αδ-SPD)

With deficiencies in both major platelet granules, are described. Additional abnormalities in the metabolism of the platelet have been reported with these syndromes.

ACQUIRED PLATELET DYSFUNCTION

Secondary qualitative defects are seen in a heterogeneous group of disorders. Partial or complete defects in either aggregation, adhesion, or the release reaction may occur separately or together in the same patient.

In stem cell defects, platelet dysfunction is common, frequently associated with thrombocytopenia. The myeloproliferative disorders have been most thoroughly studied, perhaps because of the paradox of both bleeding and thrombotic disease occurring in these diseases,

not infrequently coexisting in the same patients. Each of the specific disease, polycythemia rubra vera, thrombocythemia, and myelofibrosis, may be complicated by platelet dysfunction. The bleeding may be severe with ecchymoses, epistaxis, gastrointestinal bleeding, and excessive surgical bleeding. Frequently, the platelet count is abnormally high and the complications of bleeding and thrombosis appear to be more common in patients with excessive platelet numbers. Nevertheless, in polycythemia rubra vera the elevated hematocrit (by virtue of the increased viscosity) is a more important arbiter of thrombosis than the platelet count.

The peripheral blood film often shows platelets with great variation in size; extremely large platelets and megakaryocyte fragments are particularly characteristic. Ultrastructural abnormalities are present, and vary widely. Megakaryocytes are usually markedly increased in number in the bone marrow, and again often show morphological abnormalities, with small immature forms.

Connected functional abnormalities are prolongation of the skin bleeding time and disordered platelet aggregation pattern. 'Spontaneous' aggregation is not uncommon. Absent aggregation with adrenaline is the most frequent defect, seen in 66% of cases. Both increased and decreased sensitivity to other platelet agonists, abnormal glycoprotein patterns, disorders of arachidonic acid metabolism, and granule abnormalities have all been well documented. Frustratingly, the *in vitro* abnormalities have rarely been shown to correlate with clinical behavior. Further, the administration of aspirin to patients with polycythemia rubra vera does not appear to prevent thrombotic complications, and probably increases the risk of hemorrhage. Reduction of the platelet count to normal by chemotheraphy or [^{32}P] phosphorus is probably beneficial, and the risk of thrombosis is decreased by measures that keep the hematocrit below 45% in cases of polycythemia rubra vera.

Platelet function in acute leukemia and myelodysplastic syndromes has been less extensively studied; significant thrombocytopenia is usually the overriding problem. However, similar functional defects to those described with the myeloproliferative disease are well recognized, and at times cause a hemostatic defect in the patient with normal or near normal platelet numbers.

A wide variety of therapeutic drugs and dietary components cause platelet dysfunction, for the most part resulting in a mild hemostatic defect with easy bruising and a mild-to-moderate prolongation of the skin bleeding time. These drugs should be avoided where possible in patients undergoing surgery, in patients receiving anticoagulants, and in the presence of thrombocytopenia. The major mechanisms of drug interference with platelet function, and examples of such agents, are shown in Table 16.7.

The role of platelet dysfunction in uremia has been described earlier. In dysproteinemias, coating of the platelet membrane and thrombogenic connective tissue by the paraprotein interferes with platelet adhesion and aggregation. The defect, reflected by a prolonged skin bleeding time, is correctable by plasmapheresis.

As indicated above, platelet autoantibodies may be associated with platelet dysfunction in the absence of thrombocytopenia. Platelet activation causing degranulation, but not excessive shortening of platelet survival time, may result in an acquired 'storage pool' deficiency and may be the common mechanism of dysfunction accompanying a number of situations, which include disseminated intravascular coagulation (DIC), massive transfusion, thrombotic thrombocytopenic purpura, hamolytic uremic thrombocytopenic purpura, hemolytic uremic syndrome, cardiopulmonary by pass, and even myeloproliferative or leukemic diseases.

Table 16.7: Drugs affecting platelet function

Mechanism of effect	*Examples*	*Clinically significant defect at therapeutic doses*
Interference with arachidonate metabolism		
Phospholipase A inhibition	Meparcrine	No
Alteration of membrane phospholipid	Dietary eicosapentanoic acid (fish oil)	Yes
Cyclo-oxygenase inhibition	Acetylsalicylic acid	Yes
	Indomethacin	Yes
	Other NSAIDs	Variable
Thromboxane synthetase inhibition	Imidazole analogues	Yes
Drugs that increase cycle AMP levels directly		
Prostanoids	Prostacyclin and prostaglandin E_1	Yes
Phosphodiesterase inhibition (probably other mechanisms)	Dipyridamole, theophylline	No
Other mechanisms		
React at platelet membrane and other levels of platelet metabolism	Sulfinpyrazone	No
	Ticlopidine	Yes
	Antibiotics such as penicillin, carbenicillin (high dose)	Yes
	Dextran infusion	Yes
	Calcium channel blockers,	Probably not
	β adrenergic blockers,	Probably not
	Hydroxychloroquine, alcohol	Probably not

THROMBOCYTOSIS AND THROMBOCYTHEMIA

Thrombocytosis is defined as an increase above normal values, i.e. greater than 400×10^9/L, in the number of platelet in the peripheral blood. The causes are enlisted in Table 16.8.

Table 16.8: Causes of thrombocytosis

'Reactive' thrombocytosis
- Hemorrhage—moderate increase in the platelet count may follow acute hemorrhage
- Surgery and trauma, particularly fractures of bones
- Iron deficiency anemia
- Splenectomy
- Infection—occasionally
- Noninfective inflammatory disorders including collagen diseases
- Malignancy especially in Hodgkin's disease, carcinoma

Thrombocythemia
- Polycythemia vera and myelofibrosis
- Idiopathic thrombocythemia
- Chronic granulocytic leukemia
- Myelodysplastic syndromes

The association of a raised platelet count with a bleeding tendency may occur with the myeloproliferative disorders, polycythemia vera and myelofibrosis, while in other cases it is the only abnormality, and is termed idiopathic thrombocythemia. Some of these cases subsequently develop the typical features of polycythemia vera, or myelofibrosis.

Reactive thrombocytosis rarely requires therapeutic intervention, except for treatment of the underlying condition. Thrombocythemia, on the other hand, is a diagnosis with major implications. The distinction between the two categories is important in that patients with 'reactive' thrombocytosis should not be exposed to ^{32}P phosphorus or alkylating agents, while those with thrombocythemia are at risk from both bleeding and thrombosis

IDIOPATHIC (HEMORRHAGIC) THROMBOCYTHEMIA

Primary or essential thrombocythemia are synonyms for idiopathic (hemorrhagic) thrombocythemia, a clinical syndrome charaterized by repeated excessive bleeding, especially from the mucous membranes, and thrombotic changes, particularly in small vessels. Extreme high platelet count may be seen. It is classified as one of the myeloproliferative disorders, and its pathological features often resemble those of myelofibrosis or polycythemia vera.

Clinical Features

The disorder occurs most often in middle and older age groups. The outstanding symptom is bleeding of varying severity, and small blood vessel occlusion. Gastrointestinal bleeding is most common, but hematuria, hemoptysis, menorrhagia, and bleeding after minor trauma and surgery are also common. Spontaneous bruising occurs, and large hematomas from after only mild trauma. Petechiae are rare. Thrombosis is a common complication and particularly affects toes, feet, fingers, and the cerebral circulation, but may occur at any site. Splenomegaly is common, and the liver may be enlarged. Occasionlly, splenomegaly is absent; if Howell–Jolly bodies are present in the peripheral blood, then infarction/atrophy of the spleen is probable. There is an increase incidence of peptic ulceration and of gout.

The *bone marrow* is hyperplastic with a gross increase in megakaryocytes; hyperplasia of the myeloid and erythroid series is common. Most megakaryocytes appear normal, but often immature abnormal forms are present. Sometimes, marrow trephine shows areas of fibrosis.

Blood Picture

The outstanding feature is an increase in the platelet count, which is usually over 1000×10^9/L, and often much higher abnormalities of morphology are usual with irregular and giant forms. Defects of platelet function are present in most cases. A moderate leukocytosis, may be seen. There may be a moderate shift to the left, with 1–3% myelocytes. The neutrophil alkaline phosphatase is usually increased. The red cell picture is variable with normal values, anemia, or mild polycythemia; anisocytosis and poikilocytosis may be present.

Treatment

The incidence and severity of bleeding is broadly related to the increase in platelet count; a defect of platelet function also contributes. Treatment, therefore, is aimed at reducing

the platelet count to normal or near normal. This may be achieved by the administration of ^{32}P (a dose of 3–4 millicuries, 111–148 Mbq, is usually sufficient to reduce the platelet count in several weeks) or by the use of bulsulphan. As these agents are leukemogenic they should be reserved for use in older patients. Hydroxyurea and pipobroman have all been used with success, as in the treatment of polycythemia vera and may be preferable.

Plateletpheresis and aspirin are of some value in special situations. Splenectomy is contraindicated as it causes a further rise in platelet count and may aggravate the bleeding tendency.

Course and Prognosis

The disorder usually remains quiescent for long periods after treatment, or may run a chronic course over a number of years. The patient may die from hemorrhage or thromboss, or from the more usual complications of the myeloproliferative disorders, acute leukemia or marrow failure.

CHAPTER

17

Coagulation Disorders

The mechanism of coagulation is one of the components of the hemostatic mechanism. It comprises three separate, though related, systems, the coagulation system, the coagulation-inhibitory system, and the fibrinolytic system. Pathological disturbances may occur in any one or more of these systems and lead to a bleeding tendency or intravascular coagulation, or to a combination of two, depending on a wide variety of factors. A bleeding tendency occurs when there is a deficiency of clotting factors, inhibition of the coagulation process, or excessive activity of the fibrinolytic system.

Although hemorrhagic disorders due to abnormalities of coagulation are relatively uncommon, their early recognition and accurate diagnosis is important as there are specific forms of treatment for many of them.

BLOOD COAGULATION PHYSIOLOGY

The vital role of the coagulation mechanism is carried out by the coagulation factors. The coagulation-inhibitory and the fibrinolytic systems have the important functions of preventing accidental intravascular clotting and maintaining the patency of the vascular lumen after intravascular clotting has occurred. The interrelationship of these three mechanisms may be represented diagrammatically, as given in Fig. 17.1.

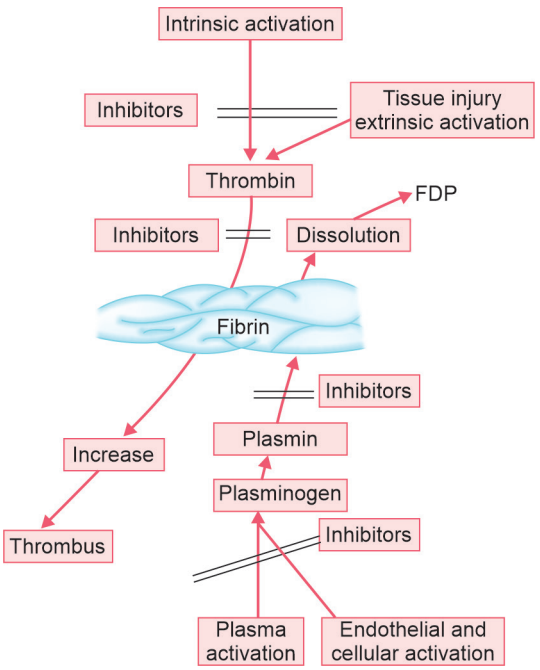


Fig. 17.1: Interrelationship between plasma coagulation, coagulation inhibitors, and the fibrinolytic system

COAGULATION SYSTEM

The main function of the coagulation system is, in the event of injury, to produce thrombin, which firstly aids the activation of platelet in hemostasis, secondly forms a stable fibrin network from circulating fibrinogen, and thirdly stimulates coagulation inactivating mechanisms, thus limiting the process to the vicinity of the injury. The components of the system are:

a. The plasma protein coagulation factors, calcium, and platelets, all of which are present in the circulating blood;

b. Certain surfaces not normally in contact with the circulating blood; and
c. Lipoprotein derived from injured tissue cells and termed 'tissue factor'.

PLASMA PROTEIN COAGULATION FACTORS

The factors and their most important features are listed below. They are present in the plasma in trace to small amounts and although difficult to isolate in pure form, they can be identified and quantitated by characterized behavior in *in vitro* tests (Table 17.1).

Factors II, VII, IX, X, XI, XII, and pre-kallikrein are inactive precursors of serine proteases, all retaining considerable homology with trypsin, which is one of the earliest evolutionary prototypes. The carboxy-terminal region is similar in all the serine proteases, consists of about 250 amino acid residues, and contains the active site region. The vitamin K-dependent factors (II, VII, IX, and X) shows considerable identity of the amino-terminal region, containing 10–12 carboxy glutamic acid residues essential for Ca^{++}-mediated phospholipid binding. The inactive zymogens are activated by limited proteolysis, often accompanied by the release of an activation peptide. Each of the active coagulation factors has a considerable degree of specificity for its substrate, this specificity being conferred by the 'binding pocket' and the conformation of the protein as a whole, which probably limits its substrate recognition.

Factors V and VII are larger glycoproteins; factor V has a molecular weight of approximately 330000 and factor VIII 360000. Activation of these factors is also achieved by limited proteolytic cleavage, resulting in the formation of two noncovalently associated peptides derived from the amino-terminal and carboxy- terminal portions of the case of factor Va

Table 17.1: Plasma protein coagulation factors

Factors		*Plasma concentration (mg/mL)*	*Bleeding disorder*		*Laboratory diagnosis*		
			Congenital	*Acquired*	*APTT*	*PT*	*TCT*
Contact*	XII	3×10^{-2}	—	—	+	–	–
	XI	$4–6 \times 10^{-3}$	Rare, mild		+	–	–
Vitamin K dependent ↑	X	1×10^{-2}	Rare, mild		+	+	–
	IX	5×10^{-3}	Uncommon, variable	Neonates liver disease and vitamin K deficiency	+	–	–
	VII	$5–10 \times 10^{-4}$	Rare, variable		–	+	–
	II	1×10^{-1}	Rare, mild		+	+	–
Antihemophilic A	VIII	$1–2 \times 10^{-4}$	Uncommon, variable	DIC	+	–	–
von Willebrand factor	V	$5–20 \times 10^{-3}$	Uncommon, variable	—	+	–	–
		1×10^{-2}	Rare, variable	DIC	+	+	–
Fibrinogen	I	2–5	Rare	DIC, fibrinolysis	+	+	+
Fibrin stabilizing	XII	8×10^{-3}	Defective wound	—	–	–	–

* Prekallikrein and high molecular weight kininogen are also involved (see text).
- Neonatal deficiency may involve all four of these factors. APTT = activated partial thromboplastin time.
PT = prothrombin time.
TCT = thrombin clotting time.
DIC = disseminated intravascular coagulation.

being of molecular weight of 94000 and 74000, respectively. There is substantial homology between the heavy and light chains of factor Va and factor Va and factor VIIIa.

von Willebrand factor forms a noncovalent complex with factor VIII, thus preventing more rapid removal of the coagulation protein from the plasma. The other contributions of von Willebrand factor to hemostasis also related to its adhesive properties, mediating adhesion of platelets to the subendothelial tissues and involvement in platelet aggregation. von Willebrand factor circulates as heterogeneous collections of oligomers ranging from the dimer of 500000 to species of greater than 10000000.

Factor I, or fibrinogen, is an asymmetrical protein consisting of three pairs of dissimilar polypeptide chains, Aα, B β_2 and γ_2 linked by disulfide bonds. The molecular weight of fibrinogen is 340000. The amino-terminl segments of all six chains are probably clustered in a central domain with fibrinopeptides A and B protruding. The proteolytic cleavage of fibrinogen by thrombin, with the release of the fibrinopeptides, is responsible for the spontaneous polymerization of fibrin molecules to form an insoluble network.

Factor XIII is an inactive proenzyme composed of two pairs of polypeptide chains ($\alpha_2\beta_2$). A platelet factor XIII consists of only two 'α' chain subunits. Factor XIII is activated to a calcium-dependent transglutaminase by thrombin cleavage of a small peptide from the amino-terminal end of the 'α' subunit. The transglutaminase activity of factor XIII is primarily directed to forming isopeptide bonds between lysine and glutamine residues of the fibrin γ chains to form γ-γ dimers, and the a chains to form polymers. These reactions, termed crosslinking or stabilization, make fibrin resistant to plasmin attack and confer structural stability.

The liver hepatocyte is the site of synthesis of most of the coagulation proteins. Vitamin K is required to convert the inactive forms of factors II, VII, IX, X in the liver to their active froms of carboxylation of amino-terminal clusters of glutamic acid residues of γ-carboxy glutamic acid. Factor VIII is probably synthesized in hepatic sinusoidal endothelial cells. von Willebrand factor is synthesized in vascular endothelium and bone marrow megakaryocytes. Plasma factor V is derived from hepatic synthesis, while platelet factor V is probably synthesized in megakaryocytes. Factor XIII is similarly disposed.

Production of these proteins is controlled by autosomal genes, with the exception of factor VIII and factor IX where sex-linked genes operate. Inherited deficiency states usually result from the production of a defective molecule, although complete or partial failure of synthesis may occur in some cases. Autosomal recessive inheritance probably explains the extreme rarity of most disorders; the disorders in which factor VIII or factor IX are inherited in the sex-linked manner (hemophilia A and hemophilia B) are relatively much more common.

Fibrinogen concentration may be measured directly as clottable protein, but the other coagulation proteins are usually expressed in units of activity compared to that of a pool of normal plasma. Immunoreactive protein levels can also be measured. It is general rule that the hemostatic efficiency of the coagulation system is not impaired unit the activity of one or more clotting factor is less than 30% of normal (0.3 U/mL).

CALCIUM

Calcium ions are essential in low concentrations for normal blood coagulation, both in the binding of coagulation factor complexes to phospholipid and in the dependency of some coagulation enzymes. When blood is collected from coagulation tests it must be prevented from clotting by the addition of a suitable concentration of a calcium-binding chemical, e.g. sodium citrate. When coagulation tests are performed on the separated plasma, an appropriate amount of calcium chloride must be added to permit clotting to proceed.

There is no evidence that coagulation disorders result from pathological reduction in ionized calcium. Nevertheless, transient prolongation of the whole blood clotting time has been observed with very rapid blood transfusion of citrated blood.

PLATELET AND LIPID (PLATELET FACTOR 3) AND MEMBRANE-BINDING SITES FOR COAGULATION FACTORS

During hemostasis, activated platelets provide negatively charged phospholipid on the membrane surface, a site for binding Ca^{++} which, in turn, binds prothrombin and the other vitamin K-dependent coagulation factors. Other specific binding sites appear on the platelet surface during the process of aggregation. Factor Va is bound to the platelet surface, providing binding sites for factor Xa. This congregation of components facilitates that local, rapid activation of prothrombin to thrombin. In coagulation tests, a substitute for platelet factor 3 is necessary, and lipids of animal or vegetable origin are commonly used.

FOREIGN SURFACES

That the blood remains fluid in the blood vessels is partly due to the fact that normal vascular endothelium does not promote blood coagulation. Foreign surfaces, both endogenous and exogenous, promote clotting in varying degrees; this appears to depend upon the surface electrical charge and the property of wettability. The inactive coagulation factor XII (Hageman factor) is activated by contact with foreign surfaces, and thus the coagulation process initiated. Coagulation may be initiated in the intravascular space by such foreign surface as tumor cells, disrupted villi as in accidential antepartum hemorrhage, and endothelial cells damaged by trauma and infarction. Potent activators include sulfate glycophospholipids and sulfatides. Some silicone surfaces and a number of plastics have a negligible or very weak effect in promoting blood clotting; this property is particularly desirable for intravenous prostheses, catheters, and extracorporeal equipment, e.g. heart-lung machines.

Earlier, a number of blood clotting tests used were insensitive or unrealiable due to the inconstant activation of the clotting process by the glass tubes; however, the addition of one of a number of surface active agents, e.g. kaolin, celite, or ellagic acid to plasma, shortly before testing, results in constant activation of the process and thus great improvement in sensitivity of the tests, e.g. activated partial thromboplastin time test.

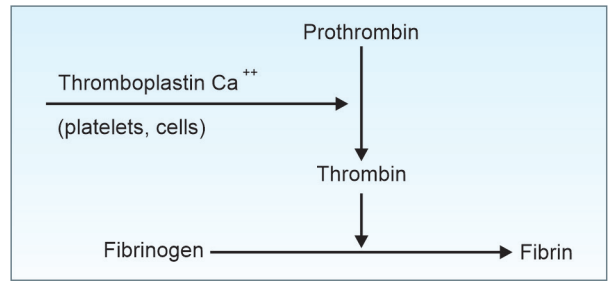
TISSUE FACTOR (TISSUE THROMBOPLASTIN)

Disrupted tissue cells expose a lipoprotein called tissue factor, which promotes blood coagulation. Induction of tissue factor expression on the surface of monocytes and vascular endothelium by a number of stimuli, including lipopoly-saccharide (endotoxin), interleukin-I, and tumor necrosis factor, has also recently been shown. This would provide a mechanism for fibrin formation in inflammatory lesions and tumors as well as after trauma.

Tissue factor consists of a single protein species, apoprotein III, in a complex with a mixture of phospholipids. The protein is oriented across the cell membrane, with a short intracytoplasmic domain. Only in combination with phospholipids, phosphatidylcholine, phosphatidylethanolamine, and phosphatidyl-serine does apoprotein III maintains its procoagulant activity. Tissue factor probably represents the cell surface receptor for factor VII. Factor VII itself is unlike the other coagulation factors in that it appears to circulate in a partially activated form. For the full expression of its activity and the initiation of coagulation, however, it requires binding to tissue factor, which, of course, is not normally exposed to the circulating blood.

THEORY OF BLOOD COAGULATION

Coagulation was formerly conceived as a process initiated by the release of an activating substance (thromboplastin) from platelets and tissue cells with a simple scheme which can be illustrated in the following ways:



With the acquisition of knowledge about individual clotting factors, the classical theory has undergone considerable changes. It is presently known that inactive prothrombin is converted to thrombin by the products formed during earlier stages of coagulation, and which can be brought about by two well-defined sets of reactions known as the extrinsic and intrinsic pathways.

REACTION SEQUENCE

A schematic diagram of coagulation sequences is shown in Fig. 17.2. A number of explanatory notes may help an appreciation of this complex system.

The mechanism of contact activation is ill understood. Surface-bound factor XII becomes susceptible to limited cleavage by kallikrein and also ultimately to auto-activation by factor XIIa itself. The same activating surfaces result in high molecular weight kininogen acting as a nonenzymatic cofactor, which interacts with both factor XII and plasma pre-kallikrein with the ultimate reciprocal activation of both. High molecular weight kininogen is also a co-factor for the activation of factor XI by factor XIIa. The contact system may activate a number of other enzyme systems, including the fibrinolytic and the kinin. Paradoxically, deficiency of factor XII, high molecular weight kininogen, and pre-kallikrein (Fletcher factor), while prolonging the coagulation times depending on the contract activation in the test tube, do not result in a clinical bleeding disorder.

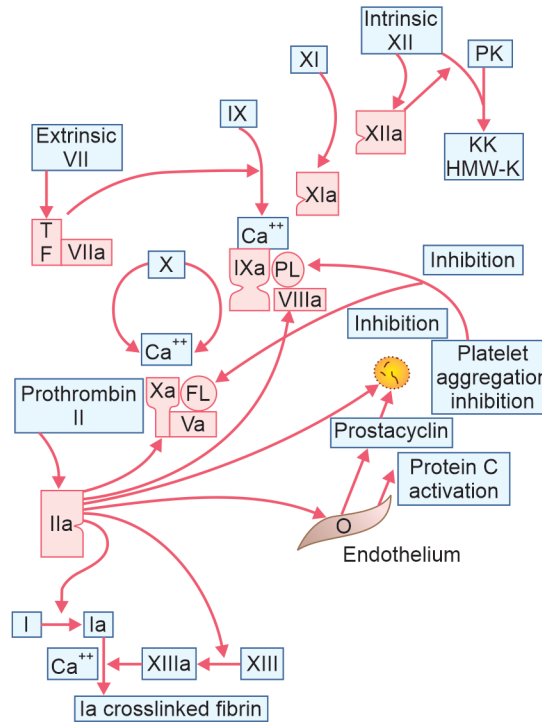


Fig. 17.2: Proposed reaction sequence in coagulation. It is of interest that components of both the kallikrein and the kinin systems are required for the effective operation of factor XII and for activation of factor XI in the test tube, and yet deficiencies in these systems do not reuslt in a clinical bleeding tendency. PK = pre-kallikrein, KK = kallikrein, HMW-K = high molecular weight kininogen, TF = tissue factor, PL = phospholipid

Factor IXa is formed by the proteolytic activation of factor IX by factor XIa in the classical '*intrinsic*' system. It is clear, however, that factor IX may also be activated by the VIIa- tissue factor complex of the 'extrinsic' system; thus the distinction between the two enzyme cascades becomes artificial. Despite the blurring of this distinction between intrinsic and extrinsic pathways, proper functioning of both is required for normal hemostasis.

Factor IXa forms a complex with factor VIIIa, Ca^{++}, and phospholipid to complete the factor X activator, termed 'tenase'. A similar complex is assembled by factor Xa, factor Va, Ca^{++}, and phospholipid to form the prothrombin activator 'prothrombinase'. The reactions are massively accelerated on the surface of activated platelet, and probably other activated cells including the vascular endothelium.

Thrombin has multiple functions which are not all shown in preceding Fig. 17.2. Note, however, the positive amplification of coagulation by thrombin activation of factor V and VIII. At the same time, thrombin activation of protein C and of vascular prostacyclin release has an opposing function to limit the coagulation process.

INHIBITORS OF COAGULATION

The naturally occurring inhibitors of coagulation provide a mechanism to limit clotting to the vicinity of tissue injury. A number of these inhibitors bear homology to the inhibitors of fibrinolysis and constitute a superfamily to protein known as 'serpins'. This group of inhibitors functions by offering their active protease an alternative high-affinity substrate that resists complete cleavage. They form a stoichiometric complex with the target protease, the complex being subsequently catabolized and cleared from the circulation. Thus the inhibitors are consumed in the process of coagulation, as are coagulation factors, and this is important when considering massive thrombosis or disseminated intravascular coagulation.

Table 17.2 lists the major inhibitors of coagulation and some of their features. A*ntithrombin III* is the major antithrombin activity, contributing about 70% of the capacity of plasma, but it also inhibits factor XIIa, XIa, IXa, and in particular Xa. Inhibition of coagulation factors by antithrombin III is greatly enhanced by the presence of heparin, primarily due to acceleration of both the cleavage and the complexing of the protease to the inhibitor. Antithrombin III is synthesized by hepatocytes and the vascular endothelium.

Heparin cofactor II selectively inhibits thrombin, and its activity is enhanced by heparin and by other proteoglycans, particularly dermatin sulfate, a major component of the vascular wall. *Alpha–2 macroglobulin* may contribute to overall anticoagulant activity, but the physiological significance is ill-understood.

Table 17.2: Blood coagulation inhibitors

Inhibitor	*Plasma concentration (mg/mL)*	*Deficiency associated with thrombosis*	*Target coagulation factor*
Serine protease inhibitors			
Antithrombin III	$1–1.5 \times 10^{-1}$	Yes	Xa, thrombin
Heparin cofactor II	–	?	Thrombin
α_2 macroglobulin	2.5	?	Thrombin, kallikrein
Protein C system			
Protein C	4×10^{-3}	Yes	Factors Va, VIIIa
Protein S	$3–4 \times 10^{-2}$	Yes	Cofactor for activated protein C

Protein C, a vitamin K-dependent protein, is a potent inactivator of factor Va and factor VIIIa when it is itself activated by thrombin with a cofactor expressed on the plasma membrane of vascular endothelium termed *thrombomodulin*. Factor Va-light chain also enhances the activation of protein C. A further vitamin K-dependent glycoprotein, protein S promotes binding of activated protein C to lipid and platelet surface, thus enhancing anticoagulant activity.

The protein C-thrombomodulin system is thus localized to the endothelium, an appropriate site to limit coagulation and thrombosis. Antithrombin III and heparin cofactor II have also the potential for local enhancement due to the glycosaminoglycan concentration in the vessel wall.

Other naturally occurring inhibitors are fibrin itself, which bind thrombin, and the breakdown products of fibrinogen and fibrin produced by the action of the fibrinolytic enzyme, plasmin. These are known as fibrin (ogen) degradation products, and have a potent antithrombin action when present in high concentrations.

PLASMA FIBRINOLYTIC SYSTEM

Physiological function of the fibrinolytic enzyme system is to digest intravascular deposits of fibrin (thrombi) in both large and small vessels and extravascular fibrin present in hemostatic plugs and in inflammatory exudates.

A simplified scheme of the fibrinolytic system is shown in Fig. 17.3.

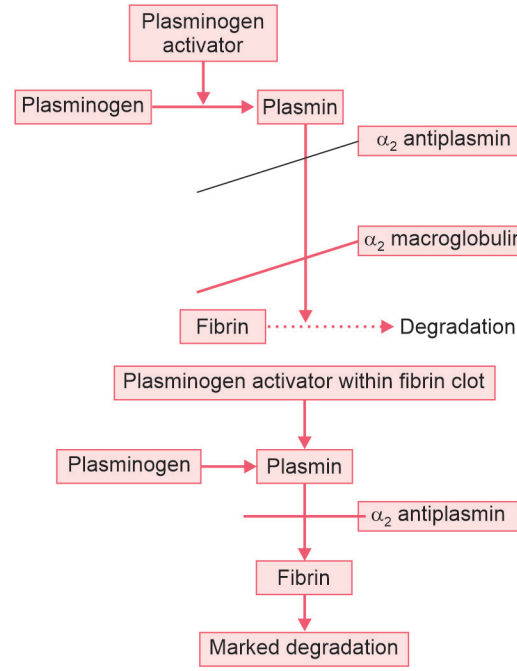


Fig. 17.3: Diagrammatic representation of the fibrinolytic system. The kinetics of the interactions are in favor of fibrin breakdown by plasmin when both activator and plasminogen are absorbed on to fibrin. The kinetics are in favor of plasmin inhibition by α_2 antiplasmin if plasminogen is activated in plasma

PLASMINOGEN–PLASMIN SYSTEM

Plasminogen is a glycoprotein of molecular weight 90000 which is synthesized in the liver. It is converted enzymatically by plasminogen activators to the fibrinolytic enzyme, plasmin. This enzyme not only digests fibrin (the desired physiological effect) but also digests fibrinogen and the clotting factor V and VIII.

ACTIVATORS

Plasminogen activator is present in the tissue (tissue plasminogen activator), in plasma, and in urine (urokinase). Tissue plasminogen activator is localized in the vascular endothelium of veins, capillaries, and pulmonary arteries, and in the microsomal fraction of cells. Tissue plasminogen activator is realeased into the bloodstream in response to a number of stimuli, including ischemia, vasoactive drugs, and exercise. Released activator is inactivated rapidly in the bloodstream by complexing to tissue plasminogen activator inhibitors, and has a half-life of about 5 minutes.

The important tissue plasminogen activator inhibitors are manufactured in the liver and in the vascular endothelium, but about 30% of the total is probably megakaryocyte-derived and is stored in platelet a granules. The activator in the urine, urokinase, differs structurally from tissue plasminogen activator, and is produced primarily in the kidneys and excreted in the urine where it may help to maintain urinary tract patency. Endothelial urokinase probably contributes a small proportion of plasma activator activity. Factor XIIa not only initiates coagulation, but also accelerates the conversion of plasminogen to plasmin via a proactivator, almost certainly kallikrein. This enzyme, together with factor XII fragments, may also react in the plasma kinin system, influencing vessel tone and permeability.

FIBRINOLYSIS

When clotting occurs, a small amount of plasminogen is trapped in the fibrin strands. Plasminogen activator, released locally from the vascular endothelium or traumatized tissues, binds to the fibrin of the thrombus and converts plasminogen to plasmin, itself bound to its substrate fibrin, and in this conformation protected from its otherwise highly effective inhibitor α_2 antiplasmin. Fibrin is thus digested. There is little or no plasma fibrinolytic activity because plasmin that is formed in the bloodstream from activation of plasma plasminogen is rapidly inactivated by circulating α_2 antiplasmin unless there is a gross excess. Alpha-2 macroglobulin also acts as a secondary plasmin inhibitor in the presence of excess plasmin.

PATHOGENESIS OF COAGULATION ABNORMALITIES

From a knowledge of the physiology of coagulation it is evident that impairment of coagulation, and thus a hemorrhagic tendency, may result from one or more of the following mechanisms.

DEFICIENCY OF ONE OR MORE BLOOD COAGULATION FACTORS

Deficiency may be due either to defective synthesis or excessive utilization with normal synthesis. Defective synthesis of the plasma protein coagulation factors results from many causes:

a. Genetic causes which usually lead to deficiency or reduced activity of a single coagulation factor;
b. Deficiency of vitamin K or its antagonism by the oral anticoagulants;
c. Severe disease of liver; and
d. Rarely in association with other diseases.

Excessive utilization of some coagulation factor occurs with intravascular coagulation and in some cases of pathological fibrinolysis.

INHIBITION OF COAGULATION BY ACQUIRED INHIBITORS

Alterations in the naturally occurring inhibitors do not cause pathological inhibition of coagulation. However, in certain circumstances, abnormal inhibitors appear and interfere with blood coagulation.

Acquired inhibitors of coagulation, although rare, are well-recognized and are usually autoantibodies with specificity for a particular coagulation factor. This is in contrast

to the naturally occurring inhibitors whose action is against the active intermediate products of coagulation. Monoclonal immunoglobins produced in multiple myeloma and Waldenstrom's macroglobulinemia may interfere with coagulation reactions, particularly fibrin polymerization, in a nonspecific fashion.

It should be kept in mind also that the fibrinogen and fibrin breakdown products which occur in acute pathological fibrinolysis are potent, though transient inhibitors of fibrin polymerization and thus blood clotting.

FIBRINOLYSIS

In a few rare conditions, large amount of tissue activator may be released into bloodstream, producing a transient but marked hyperplasminemic state. Abnormal bleeding may then occur because:

a. Fibrin which is present in wounds or hemostatic plugs is rapidly digested;
b. The products of fibrinogen and fibrin digestion (breakdown products) act as anticoagulants which interfere with fibrin clot formation and platelet function; and
c. The plasmin digests fibrinogen and factors V and VIII.

MISCELLANEOUS

Congenital and acquired disorder of platelets sometimes result in the diminished availability of platelet factor 3 *in vitro* or other platelet procoagulant activity.

In patients with primary and secondary polycythemia, abnormal bleeding occasionally complicates surgery. The bleeding probably results from an abnormally high concentration of red cells in the hemostatic plug, disordered platelet function, the effect of sludging in small vessels, and occasionally, disseminated intravascular coagulation.

DIAGNOSIS OF COAGULATION DISORDERS

A scheme for the investigation of patients with bleeding disorders, including coagulation disorders, is given later. The diagnosis of a coagulation abnormality may be strongly suspected from the clinical assessment, but requires confirmation by laboratory investigations.

CLINICAL ASSESSMENT

The significance of careful history taking and physical examination cannot be overemphasized. In a number of acquired coagulation disorders, and in some congenital ones, a presumptive diagnosis can be made from clinical features; occasionally, it is necessary to act on this diagnosis when life-threatening bleeding demands appropriate emergency treatment before the results of laboratory tests are available. Accurate clinical information is of the greatest value to the laboratory, for both the selection of tests and their interpretation.

LABORATORY INVESTIGATIONS

The laboratory investigations used in the investigation of suspected coagulation disorders belong to two groups: screening tests and special tests, including factor assays. The most commonly used tests are described in Table 17.3, and brief comments are given on their significance.

Table 17.3: Laboratory screening tests in disorders of coagulation

	Deficiency of coagulation factors								*Presence of inhibitors*							
	Prothrombin	Factor VII	Stuart–Prower	Christmas factor	Hageman	PTA	Proaccelerin	Fibrinogen	Antihemophilic	Fibrin stabilizing	Platelet number	Platelet lipid	Intrinsic prothrombin activator system	Extrinsic prothrombin activator system	Thrombin–fibrinogen system	DIC and fibrinolysis
Coagulation tests	IIa	VII	X	IX	XII	XI	V	I	VIII	XIII						
Activated partial thromboplastin time	+	–	+	+	+	+	+	+	+	–	–	–	+	+	+	+
One-stage prothrombin time	+	+	+	–	–	–	+	+	–	–	–	–	–	+	+	+
Thrombin clotting time	–	–	–	–	–	–	–	+	–	–	–	–	–	–	+	+
Clot solubility test	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
Fibrinogen/fibrin degradation products	–	–	–	–	–	–	–	–	–	–	–	–	–	–	±	+

'+ = abnormal result; – = abnormality not detected by this test. Other tests such as prothombin consumption, fibrinogen assay and thromboplastin generation may be used in some laboratories. DIC = disseminated intravascular coagulation.

Screening Tests (Fig. 17.4)

No single test is sufficiently specific to detect all types of coagulation abnormality, it is usually necessary to perform a battery of tests; most laboratories are now capable of performing a range of tests which are sufficiently sensitive to detect clinically significant abnormalities.

One-stage Prothrombin Time (Quick's Method)

This test determines the time plasma takes to clot after tissue factor (usually an extract of brain) and calcium are added. The normal range varies with the particular tissue factor used; for this reason, plasma from a normal subject must be tested at the same time. The patient's results are not regarded as abnormal unless the clotting time is more than two seconds longer then the control time. The preferred method of expression of the result is the ratio of patient to control clotting time (also called as prothrombin index). The upper limit of normal is 1.2. Currently the ratio is expressed as international normalized ratio (INR) and is the prothrombin index raised to the power by ISI of the particular kit being used.

In addition to sensitivity to prothrombin levels, the test detects deficiency of factor V, VII, X, fibrinogen, and the presence of some inhibitors. The areas of sensitivity of the major screening tests are shown. When it is necessary to distinguish between factor deficiencies and the presence of inhibitors, the test is repeated on the patient's plasma after adding a

small volume (20%) of normal plasma; the clotting time is almost completely corrected when the abnormality is due to deficiency, but is poorly corrected when inhibitors are present.

This is a very useful test for investigating coagulation abnormalities, as it is sensitive and, in general, reliable. A normal result excludes abnormality in that portion of the coagulation mechanism which the test examines; however, it is not a sensitive index of fibrinogen deficiency. Falsely abnormal results most commonly occur in patients with polycythemia; this results from the fact that the sample of the patient's plasma tested contains an excess of chemical anticoagulant, unless allowance has been made for the high hematocrit when the blood sample is first taken. Concurrent oral anticoagulant or heparin therapy may also cause an abnormal result.

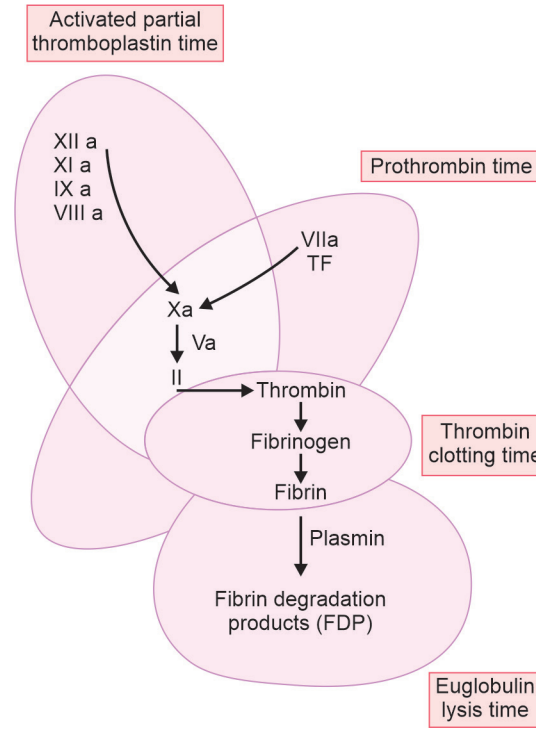


Fig. 17.4: Scheme of blood coagulation showing parts of the mechanism examined by common investigations

Activated Partial Thromboplastin Time

This test determines the time which plasma, previously incubated with kaolin or other surface active agents (which activate the surface contact clotting factor, factor XII, and XI), takes to clot in the presence of an optimum amount of platelet lipid substitute and calcium. The portion of the coagulation mechanism that is examined by this test is shown in preceding figure. Normal plasma must be tested at the same time, and usually takes 35–45 seconds to clot. Normal times vary with laboratories (may be 21–29 seconds with some kits). An abnormal result is indicated when the patient's clotting time is 10 or more seconds longer than the control time.

Interpretation of the result is made in conjunction with the results obtained in the one-stage prothrombin time test. When the latter test is normal, an abnormal result in this test usually indicates deficiency of either factor VIII or factor IX, but rare deficiencies of factors XII, XI, pre-kallikrein, or high molecular weight kininogen, and also the presence of inhibitors, must be considered.

This test is very sensitive and, in general, a normal result may be taken as indicating that there is no clinically significant deficiency of the above clotting factors. Normal results may be falsely obtained when blood samples are contaminated with tissue juices due to difficult venepuncture.

It should be kept in mind that this test does not measure platelet factor-3 activity, a platelet substitute is used in the test. A simple modification, in which kaolin is added to platelet-rich plasma without any extraneous lipid, is available for this purpose.

Thrombin Clotting Time

This test detects the time plasma takes to clot after the addition of a solution of thrombin. The concentration of thrombin solution is adjusted to clot normal plasma in 15 seconds.

When the patient's plasma takes more than 18 seconds to clot, the result is regarded as abnormal. In addition to determining the clotting time, the quality of the clot must be observed and compared with the normal clot.

Prolongation of the clotting time may result either from hypofibrinogenemia or from the presence of inhibitors, including heparin and fibrin breakdown products. Inhibitors may be suspected when the addition of the patient's plasma to normal plasma causes prolongation of the clotting time. Failure to obtaining a clot may result from complete deficiency of fibrinogen or the presence of very potent inhibitors of the thrombin-fibrinogen reaction. Distinction between these may be made by using a more potent thrombin solution.

Screening Tests for Detecting Increased Fibrinolysis

These include the whole blood clotting time and observation for clot lysis, and more specialized procedures. A simple test for fibrin degradation products, using latex particles coated with antibody to fibrinogen degradation products, has gained wide acceptance in the investigation of mild degrees of fibrinolytic activity. Recent developments include immunoassays specific for the plasmin degradation products of cross-linked fibrin. Thus, products detected by these assays must be derived from clot or thrombus, fibrinogen and its degradation products not being recognized by the antibodies employed in the system.

Special Tests

Special tests are required:

a. To identify the deficient coagulation factor.
b. To determine the concentration or activity and so degree of the deficiency.
c. To detect and quantitate immune inhibitors of coagulation.

These investigations are now easily available and can be performed in most large hospital hematology departments. They include other procedures such as the prothrombin consumption test, coagulation factor assays, and platelet function tests. Although of considerable historical interest, the thromboplastin generation test is confined to more specialized centers as specific assays are available for most coagulation factors. Some coagulation laboratories perform highly specialized procedures to clarify the nature of some inherited disorders, to detect carriers amongst the female relatives, and to assist in the management of severe bleeding in hemophilia and the inhibitor state.

GENERAL PRINCIPLES IN THE TREATMENT OF COAGULATION DISORDERS

Four main points should be considered in the management of patients with coagulation disorders; they are:

a. The treatment of the underlying cause
b. Correction of the abnormality with drugs and replacement transfusion
c. Treatment at the bleeding site,
d. General supportive measures.

An accurate diagnosis is most important, as treatment of the individual disorders differs considerably.

Treatment of the Underlying Causative Disorder

The majority of the acquired coagulation disorders are secondary to an underlying disorder, and recognition and treatment of the underlying condition often result in relief or cure of the coagulation abnormality. The disorders include:

a. Biliary tract obstruction and intestinal disease which cause malabsorption of vitamin K.
b. Liver disease in which there is impaired synthesis of coagulation factors.
c. The ingestion of oral anticoagulant drugs or administration of heparin therapy.
d. Many disorders which causes the defibrination syndrome and pathological fibrinolysis.

Correction of the Coagulation Abnormality with Drugs and Transfusion Replacement Therapy

Drugs that are useful in the correction of coagulation abnormalities are usually specific and of value only in a limited number of disorders. They include vitamin K, protamine sulphate, calcium gluconate, antifibrinolytic agents, e.g. ε-aminocaproic acid, and the kallikrein inhibitor trasylol, heparin, desamino-8-D-arginine vasopressin, androgens, and topical hemostatics such as thrombin.

Replacement Therapy

It has the object of achieving rapid correction of the deficiency of one or more coagulation factors. Factors may be given in the form of whole blood, fresh frozen plasma, or one of the various concentrates of factor VIII or factors II, VII, IX, and X. Table 17.4 enlists the types of blood products that are used and their special indications.

The broad indications for the replacement therapy are:
a. Early treatment of spontaneous bleeding episodes.
b. Established severe or prolonged wound and tissue bleeding.
c. Control of bleeding during and after surgery and trauma.

Table 17.4: Blood products used for hemostasis

Blood product	*Special properties*	*Clinical indications*
Fresh whole blood	Platelets, all coagulation factors, red blood cells	When whole blood is required in liver disease, disseminated intravascular coagulation, acute pathological fibrinolysis, massive blood replacement, hemophilia A, and when the diagnosis is uncertain in congenital bleeders
Platelet concentrates	Platelets	Thrombocytopenic bleeding
Fresh frozen plasma (FFP)	All coagulation factors	Congenital and acquired coagulation disorders
Factor VIII concentrates		
a. Low or intermediate potency, cryoprecipitate	Factor VIII and fibrinogen	Only in hemophilia A (never use in hemophilia B)
b. High potency human factor VIII, animal factor VIII are present PPSB (factors II, VII, IX, X)*	Factor VIII	Specially valuable in major surgery and when antifactor VIII inhibitors Severe bleeding in liver disease, hemophilia B, and other congenital factor deficiencies. Sometimes in the treatment of patients with factor VIII inhibitor
Fibrinogen	Factor I	Acute disseminated intravascular coagulation and acute pathological fibrinolysis in addition to other replacement therapy

*Some preparations have been found to be thrombogenic, and care is required in their use.

Long-term prophylactic replacement therapy in the inherited disorders, especially severe hemophilia A and Christmas disease, may be beneficial, but is generally not practiced in view of the storage of materials and the possible increase risk of development of inhibitors due to antibody formation.

The type of blood product indicated depends on a number of considerations, including the diagnosis of the bleeding disorder, the type and the site of bleeding, the availability of the different blood products, and the response to therapy as judged clinically and by laboratory tests.

Irrespective of the type of coagulation abnormality, acute blood loss must always be replaced with adequate amounts of blood, either as whole blood or appropriate fractions. When there is no indication of whole blood, and it is necessary to institute or maintain corrections of the clotting factor deficiency, this can usually be achieved by transfusion of fresh frozen plasma or an appropriate concentrate. Platelet concentrates may also be required if there is associated thrombocytopenia.

Concentrates of fibrinogen, factor VIII, and a mixture of factors II, VII, XI, and X (PPSB), or II, XI, and X (prothrombinex), are indicated in many bleeding episodes in patients with known deficiencies of these factors. If insufficient amounts are available, fresh frozen plasma must be used. There is also a growing tendency to encourage home therapy with concentrates, after appropriate instruction of the family. This requires careful supervision under the direction of established hemophilia treatment centers.

The amount of material which should be given at a single transfusion depends on the severity of the coagulation defects, the amount of tissue damaged, and the site of bleeding. In general, the most important factor for determining the amount to be given is the extent of tissue damage. When it is not great, as in spontaneous episodes of bleeding and following mild trauma, a single transfusion of plasma amounting to 7–10 mL/kg bodyweight usually arrests bleeding. When tissue damage is greater or bleeding is present in a dangerous site, e.g. the tongue, it is usual to give twice this amount. In major trauma, including surgery, greater correction of the coagulation abnormality is usually required, and it is then necessary to use concentrates of clotting factors to avoid fluid overload. Treatment with concentrates should be controlled with factor assays, and the amount required adjusted accordingly.

The duration of replacement therapy depends on the cause of the bleeding disorder, the severity of tissue damage, and the response to treatment. Frequently, a single transfusion arrests bleeding due to minimal tissue damage in patients with very severe coagulation abnormalities. When tissue damage is greater, and other treatment is ineffective in correcting the underlying cause, it is usually advisable to repeat the transfusion at intervals of 24 hours or less for several days.

Following major surgery, it is essential to continue correction of the coagulation abnormality until healing occurs; it is usually essential to monitor replacement therapy and adjust the dose and its frequency according to the results of laboratory tests.

The rate of administration should be rapid in order to obtain high peak blood concentration of the deficient clotting factor and thus optimum hemostatic effect.

Mild complications of plasma component infusions include pyrogenic and allergic reactions, usually transient and requiring no specific treatment. Occasionally, antihistamine or even hydrocortisone and adrenaline may be required. Potentially more sinister is the very high incidence of laboratory evidence of hepatitis in patients receiving regular component therapy. The majority of severely hemophilic patients have antibody to hepatitis B surface antigen and a smaller proportion are hepatitis B surface antigen positive.

Most have persistently elevated liver enzyme concentrations, and biopsy evidence of liver disease is frequently present in the comparatively small numbers of patients. The long-term significance of these abnormalities is not known, but clearly administration of concentrates prepared from massive donor pools should be avoided where possible in the mildly affected patient, and vaccination of unexposed patients with hepatitis B vaccine should be undertaken.

Lastly, cases of acquired immunodeficiency syndrome have occurred in hemophilic patients who have been infected with the causative virus (HIV) in contaminated plasma fractions. Most hemophilic patients receiving replacement therapy have laboratory abnormalities associated with immunodeficiency, and circulating antibodies to HIV. With donor screening for HIV and heat treatment of factor concentrates, the risk of seroconversion are now very small.

Because of the risks of infection and the expense of preparing coagulation factor concentrates, the recent successful cloning of the gene for factor VIII is tremendously advantageous. Its expression in mammalian cells indicates the possibility of recombinant DNA-factor VIII becoming available for therapeutic use in the near future.

Local Treatment at the Bleeding Site

Wounds and Mucous Membrane Bleeding

Treatment at the bleeding site is particularly important in the case of minor wounds, as appropriate treatment usually prevents prolonged bleeding and the need for replacement therapy. The measures are pressure, topical hemostatics, and immoblization.

Local pressure assists in the arrest of bleeding by raising tissue tension, and may be applied digitally, with pressure dressings and with sutures. However, hemostasis is much more certainly achieved when topical hemostatics are used in conjunction with pressure.

The most useful type of topical hemostatics are vasoconstrictor drugs, which may be used either alone or in combination with the clotting agent, thrombin. The topical application of solutions of adrenaline in high concentration (0.5–1%) has been found to be both safe and effective. Initially, a temporary adrenaline-moistened dressing is applied with pressure to the wound for about five minutes and repeated at intervals until bleeding has stopped or is greatly reduced; a permanent dressing is then applied. If bleeding tends to recur, thrombin is applied to the wound which should have been rendered relatively blood-free with vasoconstrictor drugs as the natural antithrombins in the spilled blood rapidly destroy the applied thrombin. Thrombin is usually in aqueous solution (1000 units of thrombin per mL); it may also be dissolved in the solution of adrenaline.

The usual indications for suturing wounds apply, but bleeding should be controlled with topical hemostatics; replacement therapy should be given if repeated attempts at local hemotasis fail, or in the case of a very large and deep wound.

The immobilization of wounds by bandaging, and if necessary with splinting, helps to prevent recurrence of bleeding. Unless contraindicated, wounds should remain undisturbed until healing is judged to have occurred; sutures should generally be allowed to remain in position much longer than usual.

When small wounds of mucous membrane cannot be immobilized, e.g. lacerations of the tongue, repeated application of local hemostatics usually prevents recurrence; initially, applications should be made at short intervals, and then several times a day until healing is evident.

Hematomas and Hemarthrosis

In attempting to prevent or limit bleeding, local pressure should be applied early to the site after accidental contusion or puncture; however, if bleeding progresses, pressure should not be excessively increased as it is usually ineffective and aggravates pain. Usually, bleeding into the tissues is self-limited, but continued bleeding may lead to symptoms that demand its arrest by replacement therapy.

The most useful local measures in this type of bleeding are elevation and immobilization of the part. Aspiration of hematomas is generally contraindicated unless it is certain that loculation has occurred, and then should be done by after correction of the abnormality in coagulation. Aspiration of joints is sometimes performed, although it is not universally recommended, and is largely confined to the knees. More rapid resolution of hemarthroses with earlier ambulation may be achieved, but aspiration must always be preceded by coagulation factor replacement.

General Supportive Measures and Care

While in the acute and convalescent stages of a bleeding episode, it is necessary to institute measures that aid in the arrest of bleeding, promote healing, and return the patient to normal activity. Relief of pain, anxiety, and hypovolemia aid in the arrest of bleeding. Return to normal activity is helped by the correction of iron deficiency resulting from blood loss, and by physiotherapy when indicated.

Congenital and long-standing acquired coagulation disorders require the adoption of special measures to prevent unwarranted psychological and social complications, for appropriate emergency care of acute episodes of bleeding, and for hereditary counseling in the congenital disorders. The adoption of such measures has avoided much of the invalidism once associated with severe bleeding disorders.

CLINICAL DISORDERS DUE TO COAGULATION ABNORMALITIES

The clinical disorders due to coagulation abnormalities are described under two headings—congenital coagulation disorders, and acquired coagulation disorders. The clinical manifestations of defective coagulation are, in general, similar in the different types of coagulation disorder, an indepth description of the hemorrhagic symptoms is given under the heading of hemophilia given subsequently.

CONGENITAL COAGULATION DISORDERS

Congenital coagulation disorders are rare; the most common is hemophilia A (congenital factor VIII deficiency) which has an estimated birth incidence in different countries ranging from 1 in 10000 to less than 1 in 100000.

These disorders are almost invariably due to deficient activity of a single coagulation factor, which results from a genetically determined abnormality in synthesis. In congenital hypoprothrombinemia, variant forms. von Willebrand's disease involves factor VIII deficiency similar to that occurring in hemophilia A in terms of coagulant activity, but other functions associated with von Willebrand factor and influencing platelet activity are also lacking, giving a condition with multiple hemostatic defects. There are rare instances of congenital deficiency of more than one factor, such as factors V and VIII (Table 17.5).

Table 17.5: Congenital coagulation disorders

The hemophilias
Hemophilia A (classical hemophilia)
Hemophilia B (Christmas disease)
von Willebrand's disease
Other congenital deficiency disorders
Fibrinogen (factor I) absence or deficiency
Prothrombin (factor II) deficiency
Factor V deficiency
Factor VII deficiency
Factor X (Stuart factor) deficiency
Factor XI (plasma thromboplastin antecedent) deficiency
Factor XII (Hageman factor) deficiency
Factor XIII (fibrin-stabilizing factor) deficiency
Fletcher factor (pre-kallikrein) deficiency
Fitzgerald factor (high molecular weight kininogen) deficiency

HEMOPHILIA A AND HEMOPHILIA B

Hemophilia was the first hemorrhagic disorder to be accurately described when it was recognized as an hereditary bleeding disorder of males which is transmitted by healthy women. The term hemophilia A is used to describe the disorder when factor VIII is deficient, and hemophilia B to describe the disorder when factor IX is deficient. Hemophilia A is seven times more common than hemophilia B.

Synonyms for hemophilia A are true hemophilia and classical hemophilia; those for hemophilia B are Christmas disease and plasma thromboplastin component (PTC) deficiency.

Inheritance

Hemophilia A and hemophilia B are genetically unrelated, but both are inherited as sex-linked recessive characters because the genes for the disorders are carried by the X chromosome. Thus females carrying a gene for hemophilia on one of their two X chromosomes transmit the gene to half of their female offspring and to half of their male offspring according to the laws of chance. On the other hand hemophiliacs, having only one X chromosome, transmit the gene to all their female offspring, but to none of their male children.

Males who inherit a gene for hemophilia invariably have deficiency of the corresponding coagulation factor. The concentration of the clotting factor in their plasma ranges from complete deficiency to as high as 30% of normal. Related males with hemophilia have a similar, and usually identical, concentration of the deficient clotting factor in their blood; it does not vary with age. It can therefore be concluded that in addition to the two types of hemophilia being genetically distinct, the plasma concentration of the clotting factor concerned is also genetically controlled, and that there are different grades of hemophilia.

In about 30% of cases, usually with severe hemophilia, evidence of inheritance is lacking. This is presumably due to recent gene mutation which causes the mother to become a carrier, or alternatively after several generations of carrier daughters.

Females carrying a gene for hemophilia are called carriers. A small proportion of carriers, especially of hemophilia B, have a very mild tendency to bleed and a subnormal

concentration of the relevant clotting factor in their blood. Rarely, the deficiency of the clotting factor is sufficiently severe to cause a moderate bleeding tendency. Women in families with hemophilia often wish to know if they are carriers. When tests show a discrepancy between factor VIII activity and von Willebrand factor levels (normally quantitatively related), a woman can be a suspect as a carrier of hemophilia A. The same cannot be said for hemophilia B, and in neither condition does a normal result exclude the possibility of the carrier state.

Genetic analysis using a DNA probe that detects a very polymorphic region of the X chromosome, closely linked to hemophilia A, is reported to be informative in more than 90% of families with the disease. This technique can be used in both carrier detection and in prenatal diagnosis using chorionic villous biopsy or fetal blood collection. Similar probes are available for investigation of carrier status in hemophilia B and in von Willebrand's disease.

Incidence

The frequency of hemophilia varies in different races; the highest incidence is reported in populations of British and northern European ancestry. In Australia, the incidence is 1 per 5000 male births; the reported incidence in other countries is usually lower.

Clinical Features

The bleeding tendency usually appears in infancy, sometimes during the first few weeks of life, but in mild cases it may not become apparent until adolescence or even adult life.

Severity of the Bleeding Tendency

The clinical manifestations vary in severity from patient to patient, but they tend to be the same in related hemophiliacs. The clinical severity is closely, but not absolutely, related to the concentration of the deficient factor. When the concentration of the clotting factor is less than 1% of normal, symptoms are usually severe; when it is between one and three percent, the symptoms are usually moderately severe. They are usually mild when the concentration is more than three percent of normal, except after surgery and severe trauma. The severity of the symptoms in hemophilia A and hemophilia B are similar for corresponding concentrations of the deficient clotting factor.

Variability of the Bleeding Tendency

Although the concentration of the deficiency factor remains the same in an individual, the clinical course of most hemophiliacs with severe deficiency, and some with moderate deficiency, is characterized by fluctuations in the severity of the bleeding tendency. During a period of an increased tendency to bleed, patients experience bleeding into the tissues after the slightest injury, and may even bleed spontaneously. During a phase of reduced tendency to bleed, they can withstand quite severe contusions without ill-effect. These phases vary in degree and duration; and increased tendency to bleed commonly lasts for weeks or months, and on occasions may persist for years. In contrast to tissue bleeding, wound bleeding does not appear to be less severe during a quiescent phase. The cause of the fluctuations in the bleeding tendency is unknown but is also observed in other hereditary hemorrhagic disorders. The active phases tend to be less prominent when emotional disturbances have been resolved, and also after puberty.

Type and Site of Bleeding

Wound Bleeding

It is the characteristic symptom of all hemophiliacs. It is usually slow and persists for days to weeks in spite of the presence of large clots. The onset of bleeding may be immediate, but is commonly delayed for hours or even days, particularly in mild hemophilia. Recurrence of bleeding after hemostasis has apparently occurred is particularly common.

Tissue Bleeding

Spontaneous bleeding may occur into almost every tissue of the body, but is more common in some sites than others; it occurs frequently in patients with severe deficiency, infrequently in moderate deficiency, and rarely in mild deficiency. Injuries causing contusion, ligamentous strains, or rupture of muscle fibers result in excessive bleeding at the site of injury in all but very mild hemophiliacs.

The extent of bleeding depends on the amount of tissue damaged, the concentration of the clotting factor, and the presence or absence of an active phase of bleeding. Bleeding into the tissues causes the formation of hematomas which vary in blood content from a few milliliters to several liters. The size of hematomas and the complications which arise from them are greatly reduced by early replacement therapy.

Besides pain and swelling, there may develop fever, anorexia, leukocytosis, and anemia of moderate to severe degree. Retroperitoneal and mesenteric bleeding is relatively common; in severe deficiency, intra-abdominal bleeding is a much more frequent cause of abdominal pain than is acute appendicitis.

Skin

A tendency to bruise excessively after slight injury is noted by most hemophiliacs, but spontaneous bleeding into the skin and subcutaneous tissues is common only in severe deficiency. Superficial abrasions rarely cause excessive bleeding, but lacerations and contused wounds are frequently followed by prolonged and troublesome bleeding lasting many weeks. Petechial bleeding is rare.

Mouth and Nose

Bleeding from lacerations of the tongue and the frenum of the upper lip is common in children. Bleeding from the gums is uncommon during primary dentition, but is sometimes troublesome during the shedding of these teeth. Bleeding from the sockets after tooth extraction is almost invariable, and in mild hemophiliacs is often the first and sometimes the only manifestation of the disease. In severe hemophilia, spontaneous epistaxis and bleeding into the muscular tissues of the tongue are not uncommon.

Synovial Joints

Prior to effective replacement therapy, patients with severe hemophilia suffered recurring hemorrhage into joint spaces. It is less likely in moderate hemophilia, and may never occur in mild hemophilia. Hemarthroses may occur spontaneously, but usually result from a minor joint strain or from a direct injury; they happen most frequently during an active phase of bleeding. The pain and disability of a hemarthrosis depend on the rapidity and duration of bleeding, and vary from mild to very severe; commonly, the acute symptoms persist for 3–4 days, but recovery may take weeks. In infants, the ankle joints are most commonly affected, but in older children and adults the knees are most frequently affected. The elbows are next most commonly involved, while the shoulder, wrist, hip and finger

joints are affected less frequently. The synovial joints of the spinal column are affected only rarely. Unless hemophilia has been diagnosed previously, rheumatic fever, septic arthritis, and acute osteomyelitis may be diagnosed in error. Chronic arthritis commonly develops in joints that have been the site of recurring hemorrhages.

Central Nervous System

Intracranial hemorrhage, either extra- or intracerebral, is not uncommon, particularly in severe hemophilia. In children, it occurs most commonly after head injury, but in adults it is usually spontaneous. Bleeding into the spinal cord or canal is rare.

Urogenital and Gastrointestinal Tracts

Hematuria is an occasional symptom of some patients with severe hemophilia, in whom it usually occurs without apparent cause. Bleeding usually lasts for 7–14 days, and is sometimes accompanied by ureteric colic due to the passage of clots. It is very rare in mild hemophilia, except after trauma. Hematemesis and bleeding per rectum are not uncommon, but rarely occur in the absence of other symptoms. Gastrointestinal bleeding is commonly the result of ingestion of aspirin or alcohol; occasionally it occurs as a complication of a peptic ulcer or erosion of the mucosa due to an intramural hematoma.

Complications of Hemorrhage

The incidence and severity of complications is greatly lessened by early and adequate treatment.

Pain

This is the most common and disturbing symptom of hemophiliacs, particularly those with severe disease. Local pain is due to the increasing pressure in a hematoma caused by persistent bleeding; commonly there is little difficulty in diagnosing the cause of the pain, as local swelling is usually evident. Referred pain results from the pressure effects of hematomas on peripheral nerves, and nerve roots or trunks; this may be suspected when pain is severe and local swelling is absent. Visceral-type pain most commonly results from intramural hematomas of the intestinal wall; occasionally it is due to mediastinal hemorrhage. Early replacement therapy usually produces rapid relief of referred and visceral pain—a point of importance in diagnosis.

Anemia

It frequently develops in patients with severe and moderate hemophilia, and is due to blood loss. When blood loss is acute, the anemia is normocytic in type or is slightly macrocytic due to mediastinal hemorrhage. Early replacement therapy usually produces rapid relief of referred and visceral pain—a point of importance in diagnosis.

Anemia frequently develops in patients with severe and moderate hemophilia, and is due to blood loss. When blood loss is acute, the anemia is normocytic in type or is slightly macrocytic due to the increased reticulocyte count; with chronic loss, hypochromic anemia due to iron deficiency may develop. In young children, multiple hematomas may cause temporary deviation of iron into the tissues, and can lead to the occurrence of a hypochromic anemia in the absence of blood loss. During episodes of bleeding, the hemoglobin usually falls to 7–9 g/dL but sometimes, especially with insidious bleeding, values may fall rapidly to 5 g/dL or less; this occurs particularly in small children. Hemoglobin estimations should therefore be performed daily during acute bleeding episodes, as it is not always easy to assess clinically the amount of blood lost. A moderate leukocytosis accompanies

active bleeding, especially into the tissues. When large hematomas are present, the serum bilirubin may be increased.

Constitutional Disturbances

Bleeding into the tissues and joint spaces frequently causes marked elevation of the temperature within 24 hours, which may persist for a week or more. It is usually accompanied by severe anorexia and malaise, which can persist until the hematoma resolves. These constitutional disturbances, when accompanied by a leukocytosis, may be misinterpreted as being due to a septic condition; the differentiation is sometimes difficult.

Chronic Hemophilic Arthritis

Permanent joint damage usually results from repeated hemorrhages into a joint, but may follow a single hemorrhage. The limitation of movement is usually due to fibrous adhesions within the joint, but may be due to osteophytic outgrowths. In the early stages the radiological changes are slight, but in the later stages the joint space becomes narrow and the adjacent bone ends are enlarged by osteophytic outgrowths; cyst-like rarefactions sometimes occur in the adjacent bone. In the knee joint, the intercondylar notch is enlarged; in the hips, the changes at the upper end of the femur may resemble those seen in Perthes' disease. In severe hemophilia, it is usually for several joints to become affected, but in rare cases no arthritis is clinically detectable. In the less severe grades of hemophilia, chronic arthritis is uncommon.

Pressure Effects and Sequelae of Hematomas

The most serious complications of hemophilia result from the pressure of hematomas on sensitive or vital structures. Compression of peripheral nerves is common, and results in transient or prolonged paresis, anesthesia, or hyperesthesia. The femoral nerve and the other branches of the lumbar plexus are most often affected. A condition resembling Volkmann's ischemic contracture is not an uncommon complication of a hematoma in the muscles of the forearm. Less commonly, compression of blood vessels results in gangrene of the distal part of a limb. Respiratory embarrassment is a relatively uncommon but serious complication; it may result from an obstruction of the nasopharynx by a hematoma of the tongue or from a hematoma of the larynx, due to either to blood spreading down fascial planes after tooth extraction or to local trauma. Complete intestinal obstruction due to an intramural hemorrhage is a severe but uncommon complication. Mediastinal and intrapleural hemorrhage are rare complications which may cause cardiac tamponade or respiratory failure. Recurring subcortical or medullary hemorrhage in bones leads to the formation of pseudotumors which slowly increase in size over years. Necrosis of the skin sometimes results from the pressure of an underlying hematoma; it also results from thermal and other injuries, when there is anesthesia following nerve compression.

Diagnosis

The diagnosis can usually be suspected from the clinical and hereditary features, but is established with certainty only with the aid of laboratory tests.

Clinical and Hereditary Features

The clinical features of most importance are the sex, the age of onset, and the type of bleeding; the hereditary feature of importance is evidence of sex-linked recessive inheritance. The diagnosis is therefore strongly suggested by the onset in a male child of an abnormal bleeding tendency with the characteristics of a coagulation disorder and a history

of bleeding in male relatives on the maternal side of the family. In severe hemophilia, the onset of ecchymoses, prolonged bleeding from lacerations and other wounds, and bleeding into the deep tissue and joints usually commences before the age of two years; on the other hand, in mild hemophilia symptoms may not appear until adolescence or adult life when abnormal bleeding follows tooth extraction or surgery.

As the hereditary aspects are so important, a detailed family history must be taken and a family tree drawn for accurate reference. It should be recalled that severe hemophilia frequently occurs in the absence of a previous family history of bleeding. In mild hemophilia, careful inquiry usually reveals episodes of abnormal bleeding in male relatives who do not necessarily regard themselves as being bleeders; abnormal bleeding after tooth extraction is the most common symptom.

Laboratory Investigations

The diagnosis of hemophilia A or hemophilia B requires the demonstration of deficiency of factor VIII or factor IX, respectively, in the patient's plasma. However, all the clinical and laboratory findings must be considered before the diagnosis is accepted because deficiency of factor VIII occurs also in von Willebrand's disease, and deficiency of factor IX occurs in newborn infants, liver disease, and vitamin K deficiency.

The tourniquet test is usually normal but, because of the possibility of causing a hematoma in the forearm, it is unwise to perform this test when severe hemophilia or other severe coagulation abnormalities are suspected. The skin bleeding time is usually normal; the platelet count is normal, and the platelets are morphologically normal.

Screening tests for the detection of coagulation abnormalities give the results as given below. The activated partial thromboplastin time is typically prolonged in all grades of hemophilia, except the mildest or when the patient has recently received transfusion with fresh blood, or with poorly collected blood samples. In severe hemophilia, the clotting time is usually in excess of 100 seconds, and in mild hemophilia is usually 10–20 seconds longer than the control time. The one-stage prothrombin and thrombin clotting times are normal.

Special laboratory tests should be performed in all cases to establish accurately the nature of the coagulation abnormality, as may be done with specific factor assays. In the unusual situation where these are not available, a thromboplastin generation test could be used to distinguish between factor VIII and factor IX deficiency. It is also important to exclude the presence of a coagulation inhibitor.

In hemophilic families, an early diagnosis in the neonate is often desired by mothers who are potential carriers of hemophilia. When it is known that the bleeding disorder is hemophilia A, the diagnosis can be established by using assay techniques on blood obtained from the umbilical cord at birth, or within a few days from blood obtained by heel puncture; this is possible as factor VIII is not placenta-permeable, and blood concentrations of the factor present at birth are relevant. Prenatal diagnosis is also available, using fetoscopic blood sampling and either immunoradiometric assay for factor VIII (coagulant portion) or microtechniques for factor VIII coagulant activity. Gene probes for random fragment length polymorphism using trophoblast tissue or fetal blood may shortly prove more acceptable. In the case of hemophilia B, tests should be deferred for 4–6 weeks as factor IX and other vitamin K-dependent factors may be low at birth and slow in rising to normal levels.

Prolonged bleeding rarely results when superficial veins are punctured to obtain blood specimens provided that moderate pressure is applied to the puncture site for 5 minutes; puncture of deep veins, e.g. femoral vein, and the earlobe should be avoided in patients with bleeding disorders, as prolonged bleeding may result from difficulty in maintaining pressure at these sites.

Course and Prognosis

Before the advent of blood transfusion and replacement therapy, about 90% of patients with severe hemophilia died before reaching adult life, either from exsanguination or from the pressure of a hematoma on a vital structure. Patients with moderate and mild hemophilia often died from an unrelated disease, but when death did result from bleeding, it was usually caused by severe accidental trauma or by surgery.

In severe hemophilia, several to 50 or more attacks of bleeding may occur in the course of a year; however, the severity of the attacks and their complications has been remarkably diminished with replacement therapy. Thus, the great majority of patients with severe hemophilia, and practically all with moderate hemophilia, may now expect to live well into adult life, to earn their own living, and to raise a family. The frequency of attacks of bleeding is less in those who develop appropriate attitudes.

The most dangerous hazards for a hemophiliac are intracerebral bleeding and the development of resistance to replacement therapy. The occurrence of severe headache, particularly in adult hemophiliacs, is commonly due to intracerebral bleeding; mortality is high, but is reduced when replacement therapy is commenced early. Although there is no increase in the tendency to bleed or in the frequency of attacks when immune inhibitors of coagulation develop, resistance, either partial or complete, occurs; when inhibitors are weak, it is found that increased dosage of replacement therapy is effective. However, when they are potent, replacement therapy is usually ineffective and surgery is very hazardous.

Management

Basic Aspects

Psychological Factors and Personality Development

During the past 25 years, there has been increasing realization that the bleeding tendency is aggravated by acute emotional disturbances; it is also believed by many that the course of the disorder is less severe in hemophiliacs who have developed a healthy personality and who do not identify themselves as being significantly handicapped or abnormal. A number of observations indicate that such development is influenced in childhood by parental attitudes. Mothers, in particular, experience guilt over having borne a diseased child, and may either reject or overprotect him; this commonly leads to social maladjustment, and thus failure to cope with environmental stresses. Guilt may be prevented or overcome by giving a good, not unwarranted, prognosis and by planning for her child's immediate and long-term medical care, schooling, leisure activities, and vocational training. In particular, it is important that parents avoid discouraging restrictions. When patients attend frequently with unaccountable attacks of bleeding, it is commonly found that there is an underlying emotional disturbance, the cause of which should be sought and relieved. The doctor and others who are responsible for various aspects in the upbringing of the child should take particular care to avoid measures that could directly or indirectly lead to loss of the self confidence which has been gained by earlier good management.

Schooling

By and large all severe hemophiliacs are able to attend kindergarten and ordinary schools; although most teachers are at first fearful, they too become confident with parent's assistance and the knowledge that bleeding attacks rarely result from injuries received while at school. It should be accepted by both the school authorities and the parents that any harm which might befall a child while at school is a very small price to pay for the advantages which might otherwise be denied him. Physical activities should not be

restricted, except to avoid severe injury; older children take pride in protecting, but not restricting, hemophilic children. 'Body-contact' sport, e.g. football and basketball, and games played with hard balls, e.g. cricket and baseball, should be avoided. On the other hand, even severe hemophiliacs should be encouraged to participate in active but not violent exercise, e.g. swimming, cycling, tennis, and walking; good physical condition seems to reduce incidents of bleeding from sudden accidental strains.

Vocational Guidance

As children with severe hemophilia are likely to develop some physical disabilities, education should be planned to enable them to choose a sedentary occupation, should this become necessary. However, vocational guidance to 'safe' work, rather than work in accordance with interests and abilities, leads to job dissatisfaction and sometimes aggravation of the bleeding tendency.

Emergency Medical Care

Parents should be taught that bleeding in hemophilia is not suddenly life-threatening, and how to apply local therapy until medical care is available. Appropriate medical care should be available within a few hours because, when indicated, replacement therapy is of particular value when given early. Self-administration of factor VIII concentrate, or administration by parents in the case of younger children, has dramatically reduced hospitalization, outpatient visits, and associated costs. Thus, where possible, severe hemophiliacs should be taught all aspects of self administration to cover minor hemorrhages. Hemophiliacs should be provided with a card containing details of their diagnosis for use in an event of accident; such a card is particularly important for mild hemophiliacs, as verbal statements concerning the diagnosis occasionally are not heeded by dentists and surgeons.

Special Centers

In some countries, residential schools with facilities for giving replacement therapy have been developed, or are advocated. While certain advantages are evident, they have the disadvantage of unduly emphasizing disability. Special facilities exist in many hospitals for the general care of hemophiliacs, for replacement therapy, and for other specialized care. In such hospitals, the availability of emergency care at all times is a major factor in minimizing the complications of bleeding and thus incapacity for work.

Other Procedures

Careful dental hygiene and regular dental examination are important. Prophylactic immunization, including hepatitis B, should be given early. A very fine gauge needle should be used, and pressure applied to the injection site for at least 5 minutes. When possible, medication should be given orally; intramuscular injections should be avoided unless the coagulation abnormality has been corrected by replacement therapy.

Older hemophiliacs should be warned against neglecting medical care through overconfidence or on account of social and business pressures.

Treatment of Bleeding

The basic principles in the treatment of bleeding are discussed in foregoing pages, the particular aspects that apply to management in hemophiliacs are discussed in more detail in following paragraphs.

General Supportive Measures

Even though most attacks of bleeding can be treated without admission to hospital, care in hospital is usually desirable in cases of infants and small children; this permits closer observation, relieves anxious parents of undue responsibility, and permits them to gain confidence and to be instructed in general management. While many types of bleeding, including hemarthrosis of weight-bearing joints, require treatment in hospital, the period of hospitalization should be kept as short as possible with the object of maintaining confidence and of encouraging early return for treatment in future attacks of bleeding.

Analgesics may be used to relieve pain, but not as a substitute for replacement therapy; aspirin and other drugs affecting platelet function should be avoided because of the increased risk of bleeding, particularly from the gastrointestinal tract.

Local Hemostatic Measures

Bleeding from small wounds and other accessible sites can usually be arrested with local treatment. In hemophilia, epistaxis can usually be controlled by external digital pressure applied just below the nasal bones for 5–15 minutes. If bleeding persists, adrenaline is carried to the bleeding area on a light cotton-wool pack and digital pressure is re-applied. Rarely, packing with ribbon gauze soaked in a thrombin–adrenaline mixture is required to arrest intractable bleeding; after its removal, vasocontrictor drops or spray are used for several days.

Replacement Therapy

Replacement therapy has been discussed earlier. It is now recognized that although about 5–10% of patients develop immune inhibitors, the advantages of replacement therapy are so great in the majority of patients that restrictions are not warranted when the therapeutic indications exist.

The usual complications of frequency transfused patients are commonly observed. It is especially important to observe the usual precautions in the crossmatching and administration of blood in a patient receiving repeated blood transfusions. Conservation of the veins is of the utmost importance.

Indications

The general indications for replacement therapy have been discussed earlier. The particular indications in hemophilia for the treatment of established bleeding are:

a. To prevent the extension of hematomas in sites which endanger life, e.g. the throat, chest, abdomen, and central nervous system.
b. To prevent or limit peripheral nerve and muscles damaged by hematomas, e.g. in the muscles of the forearm and in the psoas muscle.
c. To arrest prolonged bleeding from mucous membranes and wounds which cannot be otherwise controlled.
d. To arrest bleeding into tissues and other spaces which progress to the stage of causing severe pain.

Replacement therapy should be given in preference to analgesics when pain is obviously due to bleeding into tissue and joints, and for diagnostic purposes when the cause of the pain is uncertain. When replacement therapy is given early in an episode of bleeding, pain is usually relieved in an hour or two; this most certainly indicates the arrest of bleeding, as reduction in local signs usually follows. On the other hand, when replacement therapy is delayed, relief of pain and swelling is slow and may take many days. The incidence of complications is also greatly increased when treatment is delayed.

Prophylactic replacement therapy is also indicated after severe injury; particularly head injury, and also for surgery and tooth extraction.

Type of Blood Product Indicated

The different types of blood product that may be used for treatment in hemophilia A and hemophilia B are listed in foregoing pages. The products include whole blood, plasma, and concentrates containing factor VIII or factor IX. It is essential that the blood product used contains the required clotting factor and that it is present in the appropriate amount.

The choice of material to be used in treating or preventing bleeding episodes depends in part on the availability of concentrates, cryoprecipitate, or fresh frozen plasma, and on the severity of the particular bleed. There is a growing tendency to increase the amount of concentrates produced as blood components are more effectively utilized. Cryoprecipitate provides a useful alternative to plasma if high yields of factor VIII activity can be attended. More highly purified concentrates prepared by plasma fractionation are more expensive to produce and carry a greater risk of viral contamination, but have an important place in hemophilia treatment.

The stress in management must be on the earliest possible administration of plasma or concentrate after the onset of a bleeding episode. Home therapy with factor VIII concentrate provides the most efficient method of ensuring early treatment. The alternative to self- or family-administered home treatment is easy access to a treatment center where prompt attention is available.

If concentrates or cryoprecipitate are not available, fresh frozen plasma is effective and safe in most instances of spontaneous bleeding of relatively minor degree. The disadvantages of plasma are the large volumes required and the frequent occurrence of allergic reactions. Nevertheless, in acute hemarthrosis and hematomas, a dose of plasma between 7 and 15 mL/kg given over a period of 30–60 minutes often effectively arrests bleeding and relieves pain. This dose normally needs to be repeated at intervals of 12–24 hours until it is clear that the episode is controlled. Because of the risk of allergic reaction, it is advisable to give an antihistamine intravenously at the beginning of infusion, and corticosteroids should be available if required.

The indications for concentrates include:

a. Home therapy
b. Severe bleeding episodes or extensive tissue damage, and especially central nervous system bleeding
c. Failure of the expected response to plasma or severe allergic reaction
d. Prophylaxis for surgery.

Failure to obtain the expected improvement after several administrations of plasma or concentrates should raise suspicion that resistance to therapy may be due to the development of an acquired inhibitor; coagulation tests should therefore be performed soon after a dose of therapy. Such inhibitors may pose a very great problem. A number of approaches have been devised including:

a. Increasingly frequent infusions of high dose factor VIII concentrates.
b. Repeated plasmapheresis to reduce the inhibitor (antibody) concentration.
c. Infusion of factor IX concentrates, which in some cases appear to bypass the factor VIII lack and reduce the bleeding tendency.
d. The use of animal factor VIII concentrates, if available, for short-term administration

The dosage of a concentrate to be used is determined by the potency of the material and the severity of the episode being treated. Cryoprecipitate, as generally prepared, has

an activity approximating 100 units per bag; 400–600 units is the minimal dose required for a minor episode, and as much as 1000 units repeated 12-hourly may be required for more severe episodes or during surgery. Other concentrates containing factor VIII, factor IX, and related clotting factors have the concentrations shown on the container in either units of factor activity or as a plasma volume equivalent. This allows easy assessment of the dose required. As a general rule, an infusion of 1 unit factor VIII per kilogram bodyweight raises the plasma level by 0.02 U/mL. For factor IX, 1 unit concentrate per kilogram bodyweight results in a rise of 1.1 U/mL plasma.

Surgery

The availability of concentrates of clotting factors has rendered surgery in hemophiliacs almost as safe as for normal subjects. General surgery should be avoided whenever possible, but when necessary, it should be carried out in a centre that has the laboratory facilities for monitoring the response to replacement therapy. Before surgery, it is essential to perform *in vitro* and *in vivo* tests to ensure that the patient has not developed a specific inhibitor of clotting. Doses of the concentrate are usually given at 12-hourly intervals in hemophilia B; the dose must be adequate to increase the factor levels to the normal range (0.5–1.0 U/mL) during surgery and to maintain levels of 0.4 U/mL or more until wound healing is established. Very rarely, immune inhibitors appear during convalescence; in such cases, massive doses of concentrates may be required to prevent bleeding.

Minor

Minor procedures, including tooth extraction, should also be carried out in hospitals that provide special care for hemophiliacs. Usually, replacement therapy is required on a lesser scale than for general surgery, particularly when blood can harmlessly escape to the surface in the event of excessive bleeding, and when topical hemostatic measures can be applied concurrently. Occasionally, intensive replacement therapy is indicated, e.g. for lumbar puncture, because of the risk of nervous system bleeding.

Dental extraction is commonly required in hemophiliacs; various regimens have been used to prevent bleeding, including massive replacement therapy as in general surgery. Experience suggests that the following regimen is a satisfactory compromise when extraction of up to four tricuspid teeth is necessary: care in hospital, re-assurance and sedation when necessary, pre-extraction replacement therapy when plasma (12 mL/kg) or cryoprecipitate 600–800 units, local anesthesia, gentle extraction, insertion of catgut sutures, and local pressure. There should be acceptance by the doctor and the patient that moderate bleeding may occur, is without danger, and can be permanently arrested when replacement therapy is given on the seventh or eighth day after extraction.

The fibrinolytic inhibitors, ε *aminocaproic acid* (EACA) or tranexamic acid, are commonly administered concurrently to reduce factor VIII requirements. A satisfactory regimen consists of EACA taken orally in a dose of upto 1.5 g four times daily for one week, starting on the day of the extraction (dosage can be increased if needed). With this management, some patients with severe hemophilia experience no bleeding, and sockets heal without further treatment; more commonly, such patients can be discharged on the 10th day when healing of the sockets is well-advanced and recurrence of bleeding is unlikely.

Caution is necessary that no attempt should be made to arrest wound or tooth socket bleeding with very tight sutures or excessive external pressure as these procedures are usually ineffective and cause the lost blood to infiltrate tissues.

Desamino-8-D-arginine Vasopressin (DDAVP)

The plasma concentration of factor VIII increases in response to a number of physiological stimuli, including exercise, and to pharmacological agents including adrenaline and vasopressin. Plasminogen activator, and probably prostacyclin, are also liberated by vasopressin. The mechanism is unknown, but it does not appear to be due to vasoactivity nor to the release of a neuropeptide with secondary effect. The vasopressin analog DDAVP has comparatively few side effects—mild flushing and tachycardia. Administered intravenously in a dose of 0.3 μg/kg bodyweight, factor VIII activities (including von Willebrand factor) rise to 3–5 times baseline within 60–120 minutes, sufficient to treat minor bleeding or minor surgery in mild to moderate hemophiliacs and in patients with von Willebrand's disease. The dose may be repeated within a few hours, but tachyphylaxis develops. The simultaneous fibrinolytic response can be aborted with an antifibrinolytic agent given simultaneously.

Androgen Therapy

Androgen administration has been shown to be effective in elevating blood levels of proteins in other congenital deficiency states, namely hereditary angioedema and α_1 antitrypsin deficiency. A recent report indicates that the attenuated androgen danazol (600 mg/day) raises significantly the levels of factor VIII and factor IX in the small number of hemophilic and Christmas disease patients. The long-term clinical implications are awaited.

Special Therapeutic Measures

Corticosteroid Therapy

A condition resembling acute synovitis sometimes occurs after an attack of acute hemarthrosis. The administration of prednisone appears to be beneficial.

Physiotherapy

Although early replacement therapy has largely avoided the severe muscle wasting and joint deformities previously encountered, physiotherapy and splinting must be used when indicated.

Orthopedic Care

Long-standing deformities which have resulted from neglect can usually be improved or corrected with traction, splinting, and with increasing frequency, reconstructive surgery.

VON WILLEBRAND'S DISEASE

von Willebrand's disease is an inherited disorder of hemostasis which clinically resembles hemophilia. It is inherited as an autosomal dominant character (occasionally recessive) and affects both sexes. In the majority of cases, the bleeding tendency is of mild degree and is often limited to easy bruising, epistaxis—particularly with upper respiratory infections—and troublesome bleeding for up to 36 hours after minor lacerations and tooth extraction. This mild form was often difficult to diagnose with certainty in the past, and some patients were regarded as suffering from an ill-defined condition known as hereditary capillary fragility or vascular pseudohemophilia. More severely affected patients have troublesome bleeding such as menorrhagia and spontaneous bleeding disorders, as are found in severe hemophilia. These patients are at serious risk from surgery and trauma. The most important clinical features of von Willebrand's disease and mild hemophilia are compared with the simple easy bruising syndrome in Table 17.6 and the most important laboratory features of these disorders are compared in the Table 17.7.

Table 17.6: Clinical features of mild hemophilia, von Willebrand's disease, and the simple, easy bruising syndrome

Feature	*Mild hemophilia*	*von Willebrand's disease*	*Simple easy bruising*
Relative incidence	Uncommon	Moderately uncommon	Common
Sex	Males	Males and females	Mostly females
Family history of bleeding	Usual	Usual	Unusual
Inheritance	Sex-linked recessive	Autosomal dominant (or recessive)	Nil
Symptom			
Ecchymoses	Rare	Small, frequent	Small, frequent
Epistaxis	Rare	Common	Infrequent
Traumatic or surgical bleeding			
Onset	Delayed	Immediate	Usually none
Duration	Days to week	1–2 days but may recur	—
Minor lacerations	Rare	Usual	Rare
Tooth extraction	Usual	Usual	Rare
Menstrual loss	—	Excessive	Normal to mildly increased
Hematomas and hemarthrosis	Uncommon	Uncommon	Do not occur

Table 17.7: Comparison of laboratory tests in mild hemophilia A, von Willebrand's disease, and the easy bruising syndrome

Investigation	*Mild hemophilia*	*von Willebrand's disease*	*Easy bruising*
Bleeding time	Normal	Prolonged or normal	Rarely prolonged
Tourniquet test	Negative	Sometimes positive	Sometimes positive
Platelet aggregation			
ADP	Normal	Normal	Normal
Adrenaline	Normal	Normal	Abnormal
Collagen	Normal	Normal	Sometimes abnormal
Ristocetin	Normal	Abnormal	Abnormal
Prothrombin time	Normal	Normal	Normal
Activated partial thromboplastin time	Abnormal	Abnormal	Normal
Factor VIII activity	Reduced	Normal or reduced	Normal
von Willebrand antigen	Normal	Normal, reduced or absent	Normal
Factor IX activity	Normal	Normal	Normal

von Willebrand's disease is characterized by:
a. A defect of platelet function giving rise to a long bleeding time.
b. A coagulation defect due to deficiency of factor VIII activity in the plasma.

The diagnostic features are as follows:

- Prolonged bleeding time.
- Defective or absent platelet aggregation with ristocetin.
- Reduced ristocetin cofactor activity in plasma.
- Normal or decreased von Willebrand/factor VIII antigen in plasma.

The tourniquet test is often positive, and the platelet count is normal. Not all of the laboratory diagnostic features listed may be present. It may be necessary to extend the investigation to other family members to aid in diagnosis. The precision of laboratory investigations has been greatly improved with the introduction of ristocetin as an aggregating agent of platelets. Researchers have showed that this substance requires a factor, lacking in the plasma of patients with von Willebrand's disease, to cause platelet aggregation. Further advances havebeen in the use of immunodiffusion or immunoelectrophoretic techniques to assay for von Willebrand antigen. In von Willebrand's disease, factor VIII activity and von Willebrand factor classically vary together, whereas in hemophilia, factor VIII activity may be low or absent when von Willebrand factor is present in normal or increased amounts. The relationship of the two proteins is depicted in Fig. 17.5.

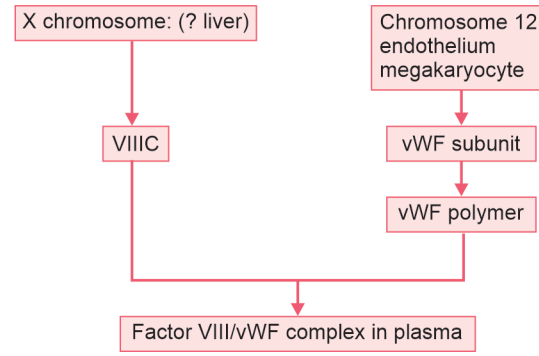


Fig. 17.5: Relationship between factor VIII and von Willebrand factor

Variant forms of von Willebrand's disease have been described, for example with normal factor VIII activity the principal abnormality being in the von Willebrand factor function affecting platelet function. The major variant (type II) of von Willebrand's disease exhibits the clinical pattern of the classical (type I) disorder. There is, however, normal factor VIII coagulation activity and quantitatively normal von Willebrand factor antigen. The ristocetin cofactor activity of this protein is abnormal, and this is related to various physicochemical characteristics which include the ability to form multimers and differences in the carbohydrate side-chains. Some authors segregate 'severe' von Willebrand's disease as type III, in part because the transmission is typically recessive. The complexity of the disease is indicated by the identification of at least 22 different subtypes on the basis of phenotype of the von Willebrand protein (Table 17.8).

Table 17.8: Varieties of von Willebrand's disease

Type I (classical)	VIII: C low vWF: Ag low vWF: Rc low
Type II A	VIII: C normal or low vWF: Ag normal or low (abnormal electrophoresis) vWF: Rc very low (prolonged BT
Type II B	As above vWF: Rc normal or low
Type III (severe)	As in type I but all activities very low or absent (recessive transmission)

VIII: C = Factor VIII activity (coagulant), vWF: Ag = von Willebrand antigen
vWF: Rc = Ristocetin cofactor, BT = bleeding time.

Correction of the various abnormalities in von Willebrand's disease, after plasma transfusion, follows a complex pattern. The bleeding time, platelet glass bead retention, and aggregation with ristocetin are corrected for a short period of time, only about 6–12 hours. The factor VIII activity, however, often increases beyond the levels explicable on the basis of the amount infused. This endogenous factor VIII activity rises to a maximum over 24 hours, and falls gradually to the resting level. The same pattern follows transfusion of normal serum or plasma from severe hemophilia A patients, both lacking factor VIII activity. The precise explanation for these findings is unknown, but may represent a stimulation to synthesize factor VIII. The von Willebrand factor functions remain clinically important, and thus the bleeding time is a better clinical parameter to monitor treatment than measurement of factor VIII.

Treatment of bleeding requires local measures and the use of fresh plasma or concentrates of factor VIII, such as cryoprecipitate. Local treatment may be sufficient for minor trauma, and the use of estrogen/progestogen preparations to regulate the menstrual cycle often helps to control menorrhagia.

OTHER CONGENITAL DISORDERS

Congenital Fibrinogen (Factor I) Deficiency

A rare disorder in which there is almost complete absence of fibrinogen in the plasma. It affects both sexes, and is inherited as an autosomal recessive character. The hemorrhagic tendency resembles that of moderate hemophilia. Bleeding most commonly follows trauma, especially severe trauma, but may occur spontaneously. Small lacerations frequently do not bleed excessively. In women, menstruation is usually normal. The characteristic laboratory finding is the failure of a clot to appear in the whole blood clotting time, the one-stage prothrombin time, and activated partial thromboplastin time and the thrombin time tests; the thromboplastin generation is normal, and assay tests show that only fibrinogen is deficient. By biochemical methods, it appears that fibrinogen is completely absent, but immunological methods may reveal trace amounts of fibrinogen. Partial deficiency of fibrinogen, hypofibrinogenemia, also occurs, probably representing the heterozygous state, and does not cause a hemostatic defect.

The general treatment is along the lines described earlier accessible bleeding points usually respond to vasoconstrictor drugs, pressure, and, if necessary, suture. Blood transfusion may be required if blood loss has been great.

Inherited Dysfibrinogenemia

A steadily increasing number of families with functionally abnormal fibrinogen has been recorded; more than 80 are now described. Only a proportion bleed excessively, some show a tendency to wound breakdown, and a very few are reported to have a thrombotic tendency.

Congenital Deficiency of Factor II (Prothrombin), Factor V, Factor VII, or Factor X

Congenital deficiency of these factors is very rare. The bleeding tendency usually commences in infancy or childhood. The disorders affect both sexes, and appear to be inherited as autosomal recessive characters. The hemorrhagic symptoms are similar to those of moderate hemophilia. Several variants of prothrombin deficiency have been described, and all have been due to the presence of a defective coagulant protein. Combined deficiency of factors V and VIII have been reported. This defect may result from an underlying lack of protein C inhibitor, allowing activated protein C to destroy circulating factors Va and VIIIa, although this notion has not been confirmed.

Factor XI Deficiency

This congenital disorder resembles moderate to mild hemophilia. It is transferred as an autosomal recessive character and affects both sexes; it is rare, and has been found most commonly in Jews. The laboratory findings include an abnormal activated partial thromboplastin time, and the deficiency is proven in a specific assay for factor XI. Without this step, the abnormality may be difficult to distinguish in the laboratory from factor XII deficiency. Bleeding is usually not severe, but may be controlled by transfusion of plasma or of the supernatant from cryoprecipitate.

Factor XII (Hageman Factor) Deficiency

Congenital deficiency of Hageman factor is characterized by the absence of a clinical hemorrhagic tendency despite prolongation of the whole blood coagulation time and the activated partial thromboplastin time. Most patients with this disorder have been discovered on routine laboratory testing. No treatment is required, and the patients may safely undergo surgical operations without special precautions. Fletcher factor (pre-kallikrein) and Fitzgerald factor (high molecular weight kininogen) deficiencies produce similar laboratory abnormalities without clinical significance, and are extremely rare.

Factor XIII (Fibrin-stabilizing Factor) Deficiency

Congenital deficiency of fibrin-stabilizing factor causes hemorrhagic symptoms similar to mild and moderate grades of hemophilia. The congenital defect has been observed in a number of families, and bleeding from the umbilical stump after birth is a characteristic feature. The healing of wounds has been stated to be less satisfactory than in other coagulation defects. The bleeding time, platelet count, and the usual coagulation tests are normal. The diagnosis is established by demonstrating that the patient's blood clot dissolves in solutions of urea or monochloracetic acid. Bleeding is treated by local measures and the transfusion of fresh frozen plasma. Factor XIII has a long plasma half-life, and plasma transfusion corrects the bleeding tendency for several days.

ACQUIRED COAGULATION DISORDERS

Bleeding due to an acquired defect of coagulation is not uncommon in clinical practice. Because the bleeding is often associated with an underlying causative disorder, it frequently occurs as a complication of a condition already under treatment by a physician or surgeon. The most common causes are liver disease, anticoagulant therapy, and vitamin K deficiency. In these disorders, the bleeding is usually of mild to moderate severity, although it is occasionally severe. However, it is of particular clinical importance as it may aggravate or precipitate bleeding from a preexisting local lesion, and may also cause bleeding to occur with minor therapeutic and diagnostic procedures, e.g. intramuscular injection and biopsy, as well as with surgical procedures.

ACQUIRED COAGULATION DISORDERS

- Vitamin K deficiency
- Liver disease
- Anticoagulant drugs
- Disseminated intravascular coagulation (DIC)
- Acute primary fibrinolysis
- Massive transfusion of stored blood

- Circulating inhibitors of coagulation recognized that disseminated intravascular coagulation contributes to the bleeding in a wide variety of disorders and, especially when associated with surgery or childbirth, may cause catastrophic bleeding.

VITAMIN K DEFICIENCY

Vitamin K is a fat-soluble vitamin which is essential for the synthesis by the liver of functional prothrombin (factor II), factor VII, factor IX, and factor X. The vitamin K-dependent step is the formation of (carboxyglutamic acid residues which are required for Ca^{++} binding associated with phospholipid binding. Vitamin K is obtained in part from food, especially green leaves, and in part from the bacterial flora in the bowel which synthesizes the vitamin; either source can compensate for a deficiency of the other. In practically all cases, deficiency of vitamin K in adults results from a clinically recognizable cause; it appears unlikely ever to be due to simple dietary deficiency. Following failure of absorption, vitamin K deficiency develops rapidly within 1–3 weeks, as the body stores are small. In practice, deficiency of vitamin K is confirmed by showing that the prolonged one-stage prothrombin time test is rapidly corrected in 6–24 hours after the parenteral administration of vitamin K.

Etiology of Vitamin K Deficiency

Vitamin K deficiency occurs in three disorders:

Disorders that Impair Fat Absorption

Obstructive Jaundice and Biliary Fistula

The main cause of vitamin K deficiency is biliary obstruction or fistula, which leads to impaired absorption due to the lack of bile salts; in long-standing obstruction, hepatic damage may develop, and thus the normal response is impaired following vitamin K administration.

Celiac Disease (Gluten Enteropathy), Pancreatic Disease, and Related Disorders

Intestinal disorders that cause malabsorption of fat and other food constituents sometimes lead to the impaired absorption of vitamin K, and thus to a coagulation disorder. In rare cases, abnormal bleeding may be the presenting symptom of these disorders.

Sterilization of the Bowel by Antibiotic Drugs

Rarely, vitamin K deficiency has been observed in patients receiving prolonged treatment with oral antibiotics. In these cases, the deficiency has been attributed to the combined effects of a diet low in vitamin K and to loss of the normal bowel flora which synthesizes the vitamin.

Hemorrhagic Disease of the Newborn

This disorder, which occurs during the first few days of life, is due to defect in the synthesis of vitamin K-dependent clotting factors. This results from one or more of the following causes: reduced stores of vitamin K, functional immaturity of the liver, lack of bacterial synthesis of vitamin K, and as a consequence of the administration of certain drugs to the mother. Thus, it has been described after the administration to the mother of oral anticoagulants, and also anticonvulsant drugs and large doses of aspirin.

Treatment

Measures used in the treatment of vitamin K deficiency are:

- Correction of the causative disorder
- Administration of vitamin K
- Replacement therapy when bleeding is severe.

Vitamin K

Many vitamin K preparations are available; they fall into two broad groups; vitamin K_1, formerly available only as a naturally occurring fat-soluble compound, but now available as a water-soluble compound; and synthetic analogues of vitamin K.

Vitamin K_1 is available in 10 mg tablets for oral administration, and in ampoules for intravenous use. An oral preparation of drops is also available.

The synthetic analogs include menaphthone , and acetomenaphthone. The synthetic analogs for intramuscular and intravenous use include menadoxime, the disphosphoric acid esters, and menadione sodium bisulfite.

Vitamin K_1 is the most potent and rapidly acting vitamin K preparation. If there is no associated hepatic dysfunction, its administration is followed by an increase in the prothrombin value above the minimal level required for hemostasis within 3–4 hours, and usually a return to normal in about 24 hours. It is therefore the preparation of choice in patients with 'hypoprothrombinemia' who are actively bleeding.

A disadvantage of vitamin K_1 is that it is relatively expensive, and thus the cheaper synthetic analogs have often been preferred when there is hypoprothrombinemia without bleeding, e.g. in obstructive jaundice and for prolonged therapy in patients with malabsorption. However, there is some doubt about the efficacy of these agents, and vitamin K_1 is being increasingly used, irrespective of whether or not bleeding is present.

The dose of vitamin K_1 for the treatment of bleeding in adults is 10–20 mg, given either intramuscularly or intravenously. When the intravenous route is used (reserved for potentially fatal hemorrhage), the drug should be diluted with blood and given slowly at a rate not exceeding 5 mg per minute. When the intramuscular route is used, vitamin K should be given through a narrow-gauge needle, and pressure applied over the injection site for at least 5 minutes. The arm rather than the buttock should be used, as bleeding is readily observed. The usual dose of the synthetic analogs given to correct hypothrombinemia is 5–10 mg three times a day, either orally or parenterally.

The response to therapy should be determined in all cases by repeating the prothrombin time test 24 hours after the commencement of treatment; failure to obtain correction or marked improvement in the prothrombin time suggests that there is hepatic disease or that absorption of the drug has not occurred if the drug has been given orally.

As prophylaxis against hemorrhagic disease of the newborn, it is not uncommon practice in many centers to administer vitamin K_1 shortly after birth to both premature and full-term infants. When there is active bleeding and the prothrombin time is prolonged, vitamin K_1 is given parenterally in a dose of 1–2 mg; this dose is repeated every 6 hours. If the bleeding is not rapidly controlled, the vitamin K_1 should be supplemented by transfusion with fresh whole blood or fresh frozen plasma. It should be noted that vitamin K_1 does not cause red cell hemolysis in infants with glucose-6-phosphate dehydrogenase deficiency (discussed in detail in earlier chapters) on the other hand, the synthetic analogues of vitamin K may produce hemolysis with possible kernicterus, and are thus best avoided in infants.

LIVER DISEASE

In liver disease, there is not uncommonly some derangement of the coagulation mechanism as shown by laboratory tests. Bleeding, when it occurs, is usually mild or moderate in degree. Troublesome or severe bleeding is relatively uncommon except:

a. When minor procedures, e.g. intramuscular injections, liver biopsy, etc. are performed;
b. When there is a local lesion, either related to the liver disease, e.g. varices, or unrelated, e.g. peptic ulcer
c. In patients with cirrhosis during and after abdominal surgery, especially shunting operations.
d. In acute fulminating hepatitis.
e. In the terminal phases of chronic liver disease, especially cirrhosis.

Occasionally, prolonged bleeding after trauma is the first sign of severe liver disease. In a patient with liver disease who is bleeding, the contribution and severity of the coagulation defect is assessed by estimation of the one-stage prothrombin time. If it is the main factor, the prothrombin time is prolonged and this is not reversed by the administration of vitamin K.

Pathogenic Factors in Bleeding

A number of factors may contribute to the hemostatic defect in liver disease. These include defective synthesis of the clotting factors, thrombocytopenia, increased fibrinolytic activity, and, rarely, defibrination. The contribution of each of these factors differs, depending on the associated clinical circumstances. However, defective synthesis is usually the most important factor.

Inhibited Synthesis of Coagulation Factors

The liver is the site of synthesis of clotting factors I, II, V, VII, IX, and X, and probably factors XI, XII, and XIII. The synthesis of coagulation factors is not equally depressed in liver disease. Thus, the activity of the vitamin K-dependent factors (II, VII, IX and X), appear to be the first to be affected; depression of factor V activity usually occurs only in severe liver disease, and hypofibrinogenemia only in very severe liver disease. Malabsorption of vitamin K due to impairment of bile salt secretion may occur in some cases of parenchymatous liver disease, and may be an additional contributing pathogenetic factor.

Thrombocytopenia

Thrombocytopenia in liver disease is usually associated with portal hypertension and congestive splenomegaly. It may, however, occur in patients with acute alcoholic liver disease in the absence of portal hypertension, and in patients with fulminating hepatitis.

Excessive Fibrinolytic Activity

The liver is the site of synthesis of plasminogen and antiplasmins. In addition, it plays an important role in clearing plasminogen activators from the bloodstream. Increased fibrinolytic activity may occur in liver disease as a result of the combined effects of impaired clearance of the plasminogen activators and decreased synthesis of antiplasmin. In practice, fibrinolysis appears to contribute to bleeding in liver disease mainly in patients with cirrhosis when subjected to surgery.

Intravascular Coagulation

The liver is the site of clearance and inactivation of some clotting factors. Inhibitors of coagulation, in particular antithrombin III and the vitamin K-dependent protein C, are synthesized in the liver, and their circulating levels may be reduced in liver disease. There is evidence for abnormally rapid consumption of coagulation proteins in cirrhotic patients. Despite these factors, intravascular coagulation is only infrequently a major factor in the hemostatic defect in patients with severe liver disease. Patients are, however, at risk from treatment with prothrombin concentrates, which have been shown in induce intravascular coagulation in some cases.

Bleeding in Hepatitis

Acute Hepatitis

Patients with acute infective hepatitis do not usually bleed abnormally and have, at most, a mild coagulation defect. Patients in whom the disease is severe usually have a prolonged one-stage prothrombin time and a prolonged activated partial thromboplastin time; these may be associated with a significant bleeding tendency, and are not corrected by vitamin K_1 administration. Patients with *acute fulminating* hepatitis usually have a marked coagulation defect, often with a severe factor V deficiency, hypofibrinogenemia, and sometimes with severe thrombocytopenia; in these patients, diffuse bleeding from skin and mucous membranes, and large hematomas, frequently occur.

Chronic Hepatitis

A number of factors, including deficiencies of coagulation factors, thrombocytopenia, and defective platelet function, may contribute to the hemostatic defects in chronic hepatitis. This defect is usually only mild to moderate, but it may aggravate bleeding from a local lesion such as esophageal varices or peptic ulcer, or it may predispose to serious surgical and post-surgical bleeding. Increased fibrinolytic activity may be an important contributing factor to bleeding when patients with chronic hepatitis undergo surgery, especially shunt operations. In addition, the hemostatic defect may be aggravated in patients who have severe gastrointestinal tract bleeding or surgical bleeding by transfusion with large volumes of stored blood.

Treatment

General Principles

Treatment of the liver disease should be commenced. The general principles of treatment of each of the possible contributing factors are as follows:

Coagulation Defect

The majority of patients do not respond to vitamin K_1 administration, but in some a slow response may be obtained after daily administration of 50 mg for 4–5 days. The coagulation defect can be improved by infusions of fresh plasma; however, this is limited because large volumes are required. Concentrates of factor II, VII, IX and X are available, but must be used with caution in patients with liver disease. These concentrates may contain activated coagulation factors and are capable of inducing intravascular coagulation. The patients may have a deficiency of antithrombin and reduced ability to clear activated factors from the circulation. The use of concentrates must therefore be followed with careful laboratory control, and it is probably advisable in most cases to use fresh frozen plasma, despite its limitations.

Thrombocytopenia

Platelet concentrates prepared from 3 to 6 units of fresh blood may be used to treat patients with severe thrombocytopenia. Unfortunately, the effectiveness of transfused platelets is reduced, because the platelets are rapidly sequestered in the enlarged spleen.

Increased Fibrinolytic Activity

This is most commonly seen in patients with chronic hepatitis during or after surgery. It may be a manifestation of disseminated intravascular coagulation, and laboratory investigation is required to establish that fibrinolysis is the major process. If fibrinolysis is considered to be the chief cause of bleeding, one of the inhibitors ε-aminocaproic acid (EACA) or aprotinin (Trasylol)-should be administered. Concurrent replacement of fibrinogen, coagulation factor, and plasma antithrombin with fresh frozen plasma, may also be advisable.

Prophylaxis

The severity of the hemostatic defect should be assessed in patients with liver disease when liver biopsy or surgery is contemplated. In the absence of bruising or bleeding, the bleeding time, platelet count, activated partial thromboplastin time, and prothrombin time serve as a useful guide to the likelihood of post-traumatic bleeding. If the partial thromboplastin time is significantly prolonged (>50 when the control range is 30–45 seconds), the prothrombin ratio (patient: control) is greater than 1.5, the platelet count is less than 100×10^9/L, and the bleeding time is prolonged, an attempt should be made to correct the defects by transfusion of plasma and/or platelet concentrates before liver biopsy or surgery is performed.

Established Bleeding

The character and degree of the underlying hemostatic defect should be assessed and then treated according to the general principles outlined above. Approximately 50% of patients with cirrhosis who bleed from esophageal varices have a demonstrable coagulation abnormality. Although this abnormality is often mild, it may nevertheless contribute to the bleeding caused by the varices and thus should be treated. When large amounts of blood are required for replacement therapy, it is important to supplement the stored blood with fresh blood and fresh frozen plasma and platelet concentrates. As with any massive transfusion, it is good practice to give fresh frozen plasma and/or platelet concentrates when large volumes of stored blood are required.

ANTICOAGULANT DRUGS

The anticoagulant drugs in clinical use are heparin and the vitamin K antagonists. Heparin inhibits the formation of thromboplastin and the action of thrombin. It is not absorbed from the gastrointestinal tract and must therefore be given by injection. Heparin has an immediate anticoagulant effect which lasts from 1 to 6 hours after intravenous injection, depending on the dose given; when given subcutaneously, the effect lasts longer because of the time taken for absorption into the bloodstream. Heparin is converted in the liver to a less active form, which is then excreted in the urine.

The vitamin K antagonists include the coumarin and indanedione derivatives. These drugs suppress an essential step in the synthesis of active vitamin K-dependent clotting factors (factors II, XII, IX and X) by the liver. The anticoagulant effect is therefore

delayed until the existing circulating clotting, factors are cleared from the bloodstream. This delay is approximately 36–48 hours with warfarin. The anticoagulant effect if warfarin and phenindione given in therapeutic doses lasts for up to two days, and that of phenprocoumon for 4 days. However, in cases of overdose the anticoagulant effect may persist even longer. The coumarins are metabolized in the liver and excreted in the urine in an inactive form. Although patients with impaired liver function show increased sensitivity to the coumarins, the biological half-life of these drugs does not appear to be increased in patients with cirrhosis. The danger of hypersensitivity reactions is considerable with phenindione, which may cause skin rashes, agranulocytosis, jaundice, diarrhea, and renal damage.

MONITORING OF ANTICOAGULANT THERAPY

Heparin therapy can be controlled by measuring the whole blood clotting time or the activated partial thromboplastin time. When heparin is given by continuous intravenous infusion, the whole blood clotting time should be maintained at 2–3 times the normal value. The corresponding prolongation of the activated partial thromboplastin time is 1.5–2.5 times the normal value. When heparin is given by intermittent injection, the clotting time should be approximately 1.5–2 times the normal value at the time the next injection is due.

Oral anticoagulant therapy is controlled either by the one-stage prothrombin time test or by the thrombotest. The therapeutic range for the one stage prothrombin time is a ratio of patient to control of 2.0–4.0 (based on the international normalized ratio), and for the thrombotest is 6–15% of normal. There are, however, local variations due to lack of standardization of laboratory technique. There is considerable individual variation in response to both heparin and the oral anticoagulants. In addition, the response to the drugs may be modified by a number of known endogenous and exogenous factors (Table 17.9).

Table 17.9: Factors interfering with control of oral anticoagulant therapy

Drugs		*Other factors*	
Enhancing	***Inhibiting***	***Enhancing***	***Inhibiting***
Salicylates	Barbiturates	Diarrhea	Hereditary resistance
Sulfonamides	Spironolactone	Hepatitis	Malignancy
Phenylbutazone	Chloral hydrate	Alcohol	
Indomethacin	Griseofulvin	Congestive cardiac failure	
Sulindac	Carbamazepine	Prolonged hypotension	
Allopurinol	Glutethimide	Septicemia	
Sulfinpyrazone	Ethchlorvynol	Acute renal failure	
Clofibrate	Cimetidine		
Thyroxine			
Oxymetholone			
Nortriptyline			
Amiodarone			
Cholestyramine			
Broad-spectrum antibiotics			

BLEEDING DURING ANTICOAGULANT THERAPY

Bleeding during anticoagulant therapy may be due to overdosage, either absolute or relative affecting response), or to a local lesion. In the best of hands, the incidence of major bleeding is very slow. Commonly, result from minor trauma or therapeutic procedures, e.g. intramuscular injection. When a person on anticoagulant therapy develops bleeding manifestations, either local or general, the laboratory test being used for control must be performed. If bleeding is confined to one site, and the result of the test indicates the anticoagulant effect is within the desired therapeutic range or is suboptimal, a local lesion predisposing to bleeding should be considered. Thus, when hematuria occurs without other bleeding manifestations, the possibility of a local renal lesion, such as renal calculus, should be considered, or if hematemesis and melena occur, the possibility of a peptic ulcer or neoplasm. However, if the tests indicates that the anticoagulant effect is greater than desired (prothrombin ration >4.0, or thrombo-test <5%), overdosage is more likely to be the cause of bleeding, especially if the bleeding occurs from more than one site.

Overdosage usually causes serious spontaneous bleeding, such as macroscopic hematuria, retroperitoneal hemorrhage, and cerebral hemorrhage, only when the coagulation tests are outside the therapeutic range for prolonged periods of time.

Increased Susceptibility

Serious spontaneous bleeding most commonly occurs when an anticoagulant drug is given in usual therapeutic dose to a patient with an increase susceptibility to the drug. Thus, it is important to be constantly aware of the factors that may alter the susceptibility to those drugs, especially in patients on long-term therapy.

Variation in susceptibility to oral anticoagulants may be caused by drugs and other factors, including a number of diseases. The drugs most commonly responsible to increase sensitivity are aspirin, the oral antibiotics, and phenylbutazone.

Increased sensitivity to the oral anticoagulants also occurs in patients with impaired hepatic function, e.g. due to hepatitis, excessive alcohol intake, congestive cardiac failure, septicemia, or prolonged hypotension, and in patients with diarrhoea, because this may be associated with impaired synthesis of vitamin K as well as impaired absorption.

Increased sensitivity to heparin may occur in patients with liver disease, severe renal disease, and oliguria, and in patients with peripheral circulatory failure.

There is an increased risk of cerebral hemorhage in patients with severe hypertension, bacterial endocarditis, and with a recent thrombotic stroke, even though the clotting tests are within the therapeutic range. Patients over the age of 65 years show an increased tendency to bleed, both because they commonly have associated arterial disease and because their dosage requirements are often low.

Accidental Overdose

Heparin is available in ampoules containing 1000, 5000 and 25000 units per mL, and accidental overdosage may occur if these doses are confused. Accidental overdosage with oral anticoagulants most commonly occurs due to confusion over tablets in the early stages of the treatment. This can be avoided by careful counselling about dosage and the use of a suitable anticoagulant book.

Deliberate Overdosage

Occasionally, bleeding results from concealed self-medication, usually in members of the medical and paramedical professions, and from suicidal or criminal poisoning.

The cause of the abnormal bleeding in patients is sometimes elicited by inquiry about drug ingestion. When drug ingestion is not admitted, it may be strongly suspected by finding deficiency of all the four vitamin K-dependent clotting factors and confirmed by analysis of the patient's plasma. Overdose of phenindione may be suspected from the presence of an orange-pink pigment in the urine and sometimes in the plasma.

Surgery and Anticoagulation Therapy

In general, surgery should not be performed while patients are on full anticoagulation therapy. When a patient on an anticoagulant requires surgery, and the anticoagulant therapy is not mandatory, the drug should be ceased, and if the surgery is urgent the appropriate antidote given. If anticoagulation is essential it may be preferable to change to heparin (either full or low dosage) during the perioperative period, as this affords the greatest control over the hemostatic defect. Many surgical procedures, however, have been carried out in patients on oral anticoagulant therapy without significant increase in hemorrhage, but great care with surgical hemostasis is obviously important. Low-dose heparin, and other antithrombotic approaches used during surgery as prophylaxis against venous thromboembolism, are discussed later.

TREATMENT

Bleeding during Heparin Therapy

It is treated by administration of protamine sulfate, a strongly basic agent which combines with and inactivates heparin. Protamine sulfate is available as an intravenous preparation in 5 mL ampoules containing 50 mg per ampoule. One milligram of protamine neutralizes approximately 1 mg (100 units) of heparin. When reversal of the effect of heparin is required within minutes of its intravenous injection, a full neutralizing dose of protamine (1 mg protamine to 100 units heparin) should be given. The effectiveness of the neutralization with protamine sulfate should be checked by estimating the clotting time or the activated partial thromboplastin time. The administration of protamine sulfate may have to be repeated because the drug is cleared from the bloodstream more rapidly then heparin. When heparin given subcutaneously has to be neutralized, protamine sulfate should be given in neutralizing dose which is equivalent to 50% of the last heparin dose, and this may have to be repeated. The exact dose of protamine required to produce neutralization test, but this test is not always available, and the approach outlined above is satisfactory in clinical practice.

Bleeding during Oral Anticoagulant Therapy

If bleeding is severe, vitamin K_1 in a dose of 25 mg should be given intravenously. Precautions should be taken to give the drug slowly, at a rate not exceeding 5 mg per minute. Rapid administration may produce flushing, vertigo, tachycardia, hypotension, dyspnea, and sweating. Reversal of the anticoagulant nevertheless takes some hours to achieve, and immediate replacement of the vitamin K-dependent factors with infusion of a concentrate or with fresh frozen plasma may be necessary. When there is no bleeding, but the prothrombin time or thrombo-test is below the generally accepted safe level and reversal is indicated, it can usually be achieved by the oral administration of 5 mg vitamin K_1 or intramuscular injection of 1–2 mg. Subsequent anticoagulation with oral agents becomes difficult if larger doses of vitamin K are used.

DISSEMINATED INTRAVASCULAR COAGULATION

Consumption coagulopathy and *defibrination syndrome* These are synonyms for disseminated intravascular coagulation, a hemorrhagic disorder in which diffuse intravascular clotting causes a hemostatic defect resulting from the utilization of coagulation factors and platelets in the clotting process. For this reason, it is so often called consumption coagulopathy. Disseminated intravascular coagulation may complicate a variety of clinical conditions as given in Table 17.10. It may be acute, subacute, or chronic.

Table 17.10: Causes of disseminated intravascular coagulation

Acute
- Obstetrical accidents
  - Abruptio placentae
  - Amniotic fluid embolism
  - Abortion
- Surgery, especially of the heart and lung
- Hemolytic transfusion reaction
- Septicemia, especially gram-negative and meningococcal
- Pulmonary embolism
- Snake bite
- Hypersensitivity reactions
- Heat stroke

Subacute or chronic
- Disseminated or localized carcinoma
- Septicemia
- Acute leukemia (particularly promyelocytic)
- Fetal death *in utero*
- Purpura fulminans
- Giant hemangioma

PATHOGENESIS

Disseminated intravascular coagulation may be caused by:

a. The release or entry of tissue factors that act as coagulants into the bloodstream; and
b. Extensive endothelial damage.

Coagulants are normally inactivated by naturally occurring circulating inhibitors, and are cleared by the reticuloendothelial system. Thus, the occurrence of intravascular coagulation is augmented by stasis (which prevent the circulating inhibitors from reaching the coagulants) and by reticuloendothelial blockade.

ETIOLOGY

Intravascular coagulation may occur as a complication of a number of disorders and clinical situations. The process may be localized or diffuse, depending on the stimulus. Clearly a number of different 'triggers' exist, and these include tissue factors and cellular material (e.g. tumors, trauma, obstetric accidents), bacterial endotoxin, and antigen–antibody complexes (mismatched transfusion). The tissue localization may be influenced by factor such as vascular tone, organ perfusion, hydration, and local inflammation.

CLINICAL FEATURES

There are two main clinical features of disseminated intravascular coagulation: bleeding, which is the most common clinical manifestation, and organ damage due to the ischemia caused by the effect of the widespread intravascular thrombosis, e.g. on the kidney and

brain. Thus, renal failure due to the small-vessel occlusion with fibrin deposits may occur in postpartum or post-surgical patients, and as a complication of septicemia. In addition, microangiopathic hemolytic anemia may occur in association with subacute defibrination states which complicate disseminated carcinoma. Occasionally, the thrombotic process affects large vessels, e.g. causes venous thrombosis and arterial thrombosis, and in these patients the thrombotic manifestations may occur with or without evidence of bleeding.

TYPE OF BLEEDING

Bleeding may be localized or generalized. Localized bleeding may take the form of prolonged bleeding from venepuncture sites, excessive bleeding at the site of operation both during operation and postoperatively, and uterine bleeding at the site of placental detachment. The generalized bleeding manifestations include ecchymoses, hematomas, gastrointestinal bleeding, and hematuria. Petechiae are often present because of the associated thrombocytopenia. Serious bleeding due to defibrination occurs most commonly as a complication of obstetrical accidents or surgery, in which it is sometimes catastrophic.

The clinical situation in pregnancy and surgery require special comment, as do the unusual occurrences of snakebite and heatstroke.

PREGNANCY

Abruptio Placentae

It is a most common cause of bleeding due to disseminated intravascular coagulation in pregnancy. The bleeding is mainly localized to the placental site; initially, it may be a concealed retroplacental hemorrhage which later becomes manifest as a vaginal bleed; generalized bleeding may also occur. The bleeding is often extensive, but usually stops as the coagulation defect undergoes spontaneous improvement within hours of delivery.

Renal failure may be a serious complication; it is considered to be due to a combination of hypotension and the deposition of fibrin in the small renal vessels.

Amniotic Fluid Embolism

It is a rare, but often fatal, condition. In a typical case, the patient develops respiratory distress and shock either during labor and delivery, or immediately after delivery. If the patient survives the initial period of shock, hemorrhagic complications are common. These may take the form of local uterine bleeding and/or generalized bleeding.

Intrauterine Death

Defibrination occurs in approximately 25% of patients in whom fetal death in utero has been present for more than one month. In most cases, there is a laboratory defect only or the patient bleed excessively from venepuncture sites; occasionally, there is a marked coagulation defect with diffuse spontaneous bleeding into the skin and mucous membranes.

SURGERY

Intravascular coagulation with severe defibrination may develop during or after any surgery, but is more common after thoracic and cardiac surgery. It may be difficult to distinguish from bleeding due to heparin used during cardiopulmonary bypass for cardiac surgery.

With thoracic surgery, intravascular coagulation is possibly due to release of thromboplastin from the lungs during surgical manipulation. Major trauma with massive

blood transfusion may also be complicated by intravascular coagulation, and laboratory assistance may be required to establish the cause of excessive and continuing bleeding.

Heatstroke with Hyperpyrexia

It is an occasional cause of acute intravascular coagulation. This results from extensive tissue damage, and may be associated with collapse and coma and acute renal failure. Cooling, fluid replacement, electrolyte balance, and general support are essential. The bleeding tendency may be treated with plasma and platelet transfusion.

The *venoms* of many different *snakes* have potent coagulation properties. Some, such as ancrod, the product of the venom of the Malayan pit viper, have a direct thrombin-like action on fibrinogen. They produce fibrin monomer formation in the circulation, which is cleared by fibrinolysis and possibly by the reticuloendothelial system, resulting in defibrination. The intermediate stages of coagulation are not influenced, and thrombocytopenia does not occur. This state is usually not accompanied by spontaneous bleeding, and ancrod has been shown to be a safe therapeutic agent in a number of thrombotic states. The majority of other venoms, act at earlier stages of the coagulation sequence and produce a state of disseminated intravascular coagulation. This can usually be managed with the appropriate antivenom, and recovery occurs rapidly after the remaining venom is neutralized.

LABORATORY DIAGNOSIS

The abnormalities in coagulation tests result from consumption of clotting factors and platelets, and the presence of circulating fibrin or fibrinogen breakdown products, resulting from the secondary fibrinolytic activity. Not all of the clotting factor activities usually consumed during coagulation are necessarily depressed in individual patients with disseminated intravascular coagulation. This is because the initial concentration or turnover rate of these various factors is subject to marked individual variations. Serial testing is therefore important in establishing the diagnosis in most cases.

The useful screening tests include observation of the whole blood clot, the thrombin time, prothrombin time and activated partial thromboplastin time, platelet count, and tests for fibrinogen–fibrin degradation products and fibrin monomers. More extensive investigation of fibrinolysis and clotting factor levels may be done if facilities are available. The major problem is to differentiate between disseminated intravascular coagulation and primary pathological fibrinolysis. The former is far more frequently the cause of severe defibrination, and is always accompanied by a detectable degree of fibrinolysis, as shown by the presence of fibrin degradation products. Primary pathological fibrinolysis is a contentious term, and may in reality represent the case in which intravascular coagulation is overwhelmed by an unusually active fibrinolytic response. This occurs in a relatively small number of clinical situations, e.g. disseminated carcinoma of the prostate.

Once the precipitating cause disappears, the clotting factor activities return to normal levels within 24 hours. Fibrinogen may show an increase earlier, although thrombocytopenia may persist for several days. The degradation products of fibrinogen and fibrin remain detectable for 12–24 hours.

TREATMENT

The principles of treatment are:

a. Elimination of the precipitating factor if possible.
b. Replacement of coagulation factors and platelets.
c. Inhibition of the clotting process with heparin or other agents.

ELIMINATION OF PRECIPITATING FACTOR

The precipitating cause is often self-limiting. Thus, the stimulus to disseminated intravascular coagulation usually disappears soon after surgery or after delivery of patients with abruptio placentae. However, some of the underlying causes require specific treatment, e.g. antibiotics for septicemia, estrogens for carcinoma of the prostate, radiotherapy and steroids for patients with giant hemangioma.

Replacement of Coagulation Factors and Platelets

Whole blood transfusion is given first to replace blood loss and second to replace the coagulation factors and platelets. If available, blood collected less than 12 hours previously may be used. However, such fresh blood is often not readily obtainable, and platelets and labile coagulation factors V and VIII, as well as fibrinogen and antithrombin, can be replaced with platelet concentrates and fresh frozen plasma. Fibrinogen may also be given in concentrated form, especially when bleeding is severe or does not respond to the above measures, and when the laboratory tests of thrombin time and fibrinogen level indicate severe deficiency. The dose is 5–10 g in 500 mL infused over 2–3 hours. The likelihood of hepatitis after fibrinogen administration is less if the material has been prepared from donor plasma known to be free of hepatitis-associated antigen (HbsAg).

Inhibition of the Clotting Process

The use of heparin or the enzyme inhibitors aprotinin (Trasylol) and ε-aminocaproic acid (EACA) should be considered in any continuing episode of disseminated intravascular coagulation. Heparin is the agent most widely used, but a place exists for the use of aprotinin and perhaps EACA.

The clear indication for heparin is the occurrence of thrombotic manifestations, which may present as organ failure or as a large vessel occlusion. Heparin, administered in an attempt to interrupt the underlying coagulation process *per se*, has not been shown to improve the high mortality associated with this condition. On the contrary, the added risk of bleeding is not inconsiderable.

Treatment should be carefully monitored. The control is best based on the thrombin clotting time, platelet count, fibrinogen level, and prothrombin time, fibrinogen level, and activated partial thromboplastin time. When heparin is required, these tests are usually all abnormal, and some further prolongation of clotting times may result after heparin is started. The required dose of heparin is usually less than that necessary to treat patients with overt thrombosis. The average requirement is approximately 1000 units per hour by continuous intravenous infusion, but patients with hepatitis or renal insufficiency, or those in circulatory failure, may be very sensitive to heparin and should be treated initially with a dose of 500 units per hour. When effective, the response to heparin is fairly rapid. The thrombin clotting time and partial thromboplastin time may shorten somewhat, but one of the best guides to successful treatment is a significant increase in fibrinogen levels within 12 hours.

The enzyme inhibitor, aprotinin (Trasylol), may also be useful in the treatment of disseminated intravascular coagulation and hyperfibrinolysis. It may be considered as an alternative to heparin or used in combination with the anticoagulant in severe or resistant cases. Similarly, ε-aminocaproic acid (EACA) may sometimes be used in combination with heparin and replacement therapy. Care should be taken to establish that fibrinolysis is a major element in the hypofibrinogenemia when inhibitors are used, as inhibition of compensatory local fibrinolysis associated with intravascular coagulation may aggravate the thrombotic tendency.

HEMORRHAGE AND BLOOD TRANSFUSION

Hemorrhage resulting from blood transfusion may be caused by:
a. The administration of large amounts of stored blood
b. Hemolytic transfusion reactions
c. Transfusion thrombocytopenia due to platelet alloantibodies.

Of these, the first is not uncommon, the second is rare, and the third is very rare.

BLEEDING AFTER TRANSFUSION OF LARGE AMOUNTS OF STORED BLOOD

Platelets and the labile clotting factors V and VIII are unstable in blood stored at 4°C. Thus, when a patient's blood volume is replaced by large amounts of stored blood, or packed red cells and plasma expanders such as albumin solutions, thrombocytopenia and deficiencies of factors V and VIII may develop because of the dilution factor. The severity of the resultant hemostatic defect is related to several factors, including the amount of blood transfused and its rate of transfusion, the period of time that the blood has been stored, and the underlying clinical circumstances.

Amount

Thrombocytopenia regularly occurs when more than 10 units (5000 mL) of stored blood is administered over a 48-hour period. If the blood is given more rapidly, or if larger volumes are given, abnormal bleeding and severe thrombocytopenia may occur. Thrombocytopenia appears to be caused mainly by dilution of the recipient's blood with platelet-poor stored blood, but it is possible that blood loss and other factors may also contribute. The platelets return to normal in about 3–5 days after the last transfusion. The levels of factors V and VIII are variably depressed, commonly to 20–30% of normal.

AGE OF BLOOD

Blood which is less than 24 hours old still contains significant amounts of factors V and VIII, and some viable platelets. However, the platelet count rapidly falls, and the level of the clotting factors appreciably declines, in blood stored for 24 hours or more. Clearly, plasma expanders and packed red cells are devoid of coagulation factors and platelets.

Circumstances Requiring Blood Transfusion

The severity of the hemostatic defect produced by transfusion with large volumes of stored blood is more marked when the capacity to produce platelets or clotting factors is impaired, e.g. in bone marrow depression, liver disease, and the hemophilias, or when the rate of consumption of platelets or clotting factors is increased, e.g. after major trauma, in chronic idiopathic thrombocytopenic purpura, and in intravascular coagulation.

Citrate overdosage may act as a minor contributing factor to the coagulation defect in massive transfusion. Thus, abnormal in vitro clotting tests corrected by the addition of extra calcium have been reported in patients who have bled abnormally after transfusion with large volumes of blood. Patients with liver disease are especially vulnerable to the hypocalcemic effects of transfusion with citrated blood because citrate is normally metabolized in the liver.

Treatment

The coagulation defect produced by transfusion with large volumes can be prevented or minimized if 2 units of fresh blood, or 3–5 units of fresh frozen plasma, are given with every 10 units of packed red cells that is rapidly transfused. Established bleeding caused by transfusion of large amounts of stored blood is treated by administration of fresh

frozen plasma, platelet concentrates or fresh whole blood. In addition, hypocalcemia can be prevented by the injection of calcium gluconate.

HEMORRHAGIC DISORDERS DUE TO CIRCULATING INHIBITORS OF COAGULATION

Circulating inhibitors are antibodies almost always of the IgG heavy chain class, with activity directed against a coagulation protein. There are two major types of inhibitor: Those occurring in the course of hemophilia or other congenital coagulation disorders, and those acquired spontaneously or in the course of some other disease state. The nature of the reaction between inhibitor and clotting factor is complex, but the effect is to inactivate the coagulant protein partially or completely. Thus, inhibitors may be detected because of the property of the patient's plasma to induce a coagulation abnormality in mixtures with normal plasma. The specificity of the reaction can then usually be established by coagulation factor assays.

Inhibitors in Hemophilia

These occur in 5–20% of the patients. Some are less potent and become undetectable after periods without treatment. Others are very active anticoagulants which persist indefinitely and pose great problems in the treatment of bleeding episodes. Massive doses of factor concentrates may be required; sometimes species specificity is present and a temporarily good response may be obtained with porcine or bovine factor VIII. Immunosuppressive treatment combined with massive factor replacement has been reported to be successful in a small number of cases. An innovation that proved helpful in about 50% of bleeding episodes is the use of concentrates of the prothrombin complex in hemophiliacs with inhibitors against factor VIII. These concentrates in some way bypass the coagulation defect to a degree sufficient to control bleeding. Commercial preparations have been produced specifically for this purpose. Immunodepletion by plasmapheresis and suppression of antibody activity by high-dose intravenous gamma globulin have been reported to be successful in small numbers of patients.

Acquired inhibitors most commonly occur in disseminated lupus erythematosus. The 'lupus' inhibitor does not normally cause a bleeding tendency. In fact, paradoxically, the condition is not infrequently associated with a thrombotic tendency. The inhibitor is detected during routine screening because of prolongation of clotting times in both the activated partial thromboplastin and prothrombin time tests.

Other acquired inhibitors have been described against most of the coagulation factors, but are rare occurrences. The most frequently encountered of these have activity against factor VIII or its von Willebrand factor component, and have been described in association with penicillin reactions, pregnancy, rheumatoid disease, and spontaneously in the elderly, sometimes in association with skin disorders.

Factor VIII inhibitors may result in a bleeding tendency similar to mild or severe hemophilia. Menorrhagia is sometimes severe, and occasionally hemarthrosis, and retroperitoneal or gastrointestinal bleeding occur. When associated with pregnancy, bleeding occurs within a few weeks to several months of childbirth. The inhibitor usually disappears, but has been described to recur with subsequent pregnancies. Passive transfer across the placenta to the fetus has been observed.

Treatment

Treatment of acquired inhibitors is often unsatisfactory. Remission of the underlying disorder such as lupus usually results in loss of the inhibitor. When bleeding occurs, blood transfusion and large doses of a concentrate of the appropriate factor, together with immunosuppressives, may control hemorrhage.

OUTLINE OF INVESTIGATIONS OF A PATIENT WITH A BLEEDING TENDENCY

While trying to diagnose a patient with abnormal bleeding, three questions must be answered:

1. Is the bleeding due to a local pathological lesion, a hemorrhagic disorder, or a combination of the two?
2. If due to a hemorrhagic disorder, which of the three components of the hemostatic mechanism is affected: the platelets, the blood vessels, or the coagulation mechanism? Is more than one component affected?
3. What is the cause of the hemorrhagic disorder?

The importance of the history and clinical examination must be emphasized because the diagnosis of many hemorrhagic disorders is largely or wholly clinical, and the selection of appropriate laboratory tests required for accurate diagnosis depends on the full clinical assessment.

The screen highlighted text given below summarizes the clinical features that should be sought and the special tests which may be necessary. In most cases, an adequate history and physical examination together with a few relatively simple investigations will establish the cause of the disorder.

IS THE BLEEDING DUE TO LOCAL PATHOLOGICAL LESION, A HEMORRHAGIC DISORDER, OR A COMBINATION OF THE TWO?

This question can often be answered from a consideration of the type of bleeding. Careful questioning about past bleeding and consideration of the existence of predisposing conditions may give valuable information. Thus, as pointed out previously, hemorrhagic disorder should be suspected:

a. When there is spontaneous bleeding into the skin, mucous membranes, or interstitial tissues.
b. When there is excessive or prolonged bleeding after minor trauma or minor surgery.
c. When the bleeding occurs from more than one site.

Furthermore, a hemorrhagic disorder may be suspected when there is evidence of a clinical disorder that commonly causes bleeding, or when there is a family history of abnormal bleeding.

It should be realized, however, that although bleeding from more than one site is usual in a hemorrhagic disorder, occasionally an episode of bleeding is localized to one site.

It should also be remembered that abnormal bleeding from a local pathological lesion may be precipitated by an unsuspected hemorrhagic disorder, and that in a patient with a known hemorrhagic disorder bleeding may be precipitated by the development of local pathological lesion.

DUE TO A HEMORRHAGIC DISORDER, WHICH OF THE THREE COMPONENTS OF THE HEMOSTATIC MECHANISM IS AFFECTED?

The affected component can sometimes be suspected from the type of bleeding. Thus, in platelet disorder, petechial bleeding is common, ecchymoses tend to be numerous but usually not larger than 2 cm in diameter, and bleeding from mucous membranes is prominent; furthermore, bleeding is commonly spontaneous. When excess bleeding occurs from wounds, it commences immediately, persists for less than 48 hours, and rarely recurs.

In the coagulation disorders, petechial hemorrhage is rare. Ecchymoses tend to be larger than in the platelet and vascular disorders, and bleeding more.

Investigations of a patient with hemorrhagic disorder—in brief frequently occurs into the deep tissues. Bleeding occurs commonly after minor trauma or surgery, and is less often spontaneous. Wound bleeding tends to commence after a delay of several hours, to persist for more than 48 hours, and to recur after hemostasis has apparently occurred.

History

Full general medical history with special emphasis on the following points:

Age, Sex

Present Episodes of Bleeding

Type of bleeding petechiae, ecchymoses, hematoma, deep tissue or joint bleeding, wound hemorrhage, menorrhagia, mucous membrane bleeding.

Frequency and Duration

Apparent cause spontaneous or following minor trauma or surgery

Coexisting Disease

- Disorders that may cause vascular bleeding
- Disorders that may cause thrombocytopenia
- Disorders that may cause coagulation defects
- Gastrointestinal disease
- Renal disease, particularly advanced
- Liver disease and splenomegaly (hypersplenism)
- Primary hemopoietic disorders
- Other possible associations, e.g. pregnancy, allergic reactions, skin disorders

Drug Ingestion

- Aspirin
- Nonsteroidal anti-inflammatory agents, e.g. sulfinpyrazone

Anticoagulant Administration

- Warfarin
- Phenindione
- Dietary changes or gastrointestinal upsets with vitamin K deficiency
- Other drug treatment, e.g. salicylate

Occupation

- Exposure to drugs or chemicals
- Hazards of trauma

Diet

- Ascorbic acid intake

Past History of Bleeding and Trauma

- Especially important in cases of recurrent bleeding and suspected congenital hemostatic defects:
- Age at occurrence of first abnormal bleeding and details of incident

Hemorrhagic Incidents

- Ecchymoses—traumatic and/or spontaneous, size
- Hematomas—causes, size, and duratioin
- Petechial hemorrhages
- Epistaxis—cause, severity, frequency
- Minor wound bleeding—immediate or delayed onset, rate of loss, duration, recurrences, measures required to arrest bleeding
- Melena, hematemesis, hematuria, and hemoptysis—cause and severity
- Menstrual bleeding and postpartum bleeding—severity and duration of bleeding, loss of clots, duration of blood staining of lochia, inability to carry out usual occupation during menstruation, hemorrhage associated with delivery.

Bleeding after Trauma and Surgery

Tooth extraction, tonsillectomy, circumcision, major surgery, and accidents: record all incidents and whether bleeding occurred. Time of onset of bleeding, total duration, severity, and recurrence.

Therapeutic Measures and Response

Blood transfusions, wound-suturing, cautery, pressure bandages, splenectomy, corticosteroids, vitamin K

Family History of Bleeding

- Draw family tree and enter details; interview older relatives
- Bleeding episodes in siblings and children
- History in antecedents, both paternal and maternal
- Racial and geographic origins
- Obtain results of investigations carried out on relatives with a positive history of bleeding

Examination

Complete physical examination with special emphasis on:

General	Cushingoid, myxedematous, appearance of plethoric, icteric, or cachectic patient appearance. Distribution of skin hemorrhages, contour, and mobility of limbs and trunk
Skin	Telangiectases (spider, cavernous, punctate), hemangiomas, petechiae, urticaria, ecchymoses. Texture and elasticity of skin, scars. Palms of hands
Mouth	Petechiae, lacerations, telangiectases, superficial vessel bleeding, hematomas
Wounds	Excessive blood clot, degree of healing, nature of scars
Abdomen	Superficial venous engorgement, hematoma in abdominal wall, hepatomegaly, splenomegaly, abdominal masses (intra- and retroperitoneal), ascites
Pelvis	Rectal and vaginal examination (if indicated)
Nervous system	Fundus oculiretinal hemorrhages, papilledema Peripheral nerves—sensory and motor
Joints	Swelling, tenderness, and deformity
Urine	Proteinuria, hematuria, and hemoglobinuria
Tourniquet test	

Special Investigations

Essential investigations for all cases

- Full blood examination
- Hemoglobin
- Red cell morphology
- White cell count
- Platelet count and examination of blood film for number, morphology, and presence of platelet clumping

Skin Bleeding Time

Further investigations that may be required

The further tests that may be indicated vary with disorders suspected after clinical assessment and the screening blood examination.

a. Screening tests of blood coagulation, including activated partial thromboplastin time and prothrombin time. These detect any of the important congenital or acquired abnormalities in coagulation. More elaborate procedures, such as factor assays and tests for inhibitors, may be necessary.
b. Tests of platelet function, including adhesiveness and aggregation when a qualitative disorder is suspected.

Although one may suspect the component involved from the type of bleeding, it can usually be determined with certainty only after blood examination and consideration of the other clinical features. In thrombocytopenia, the platelet count is reduced, the tourniquet test is commonly positive, and the bleeding time is usually prolonged. In coagulation

disorders, one or more of the clotting tests is abnormal. In vascular disorders, the platelet count and clotting tests are normal. Diagnosis of vascular disorders is usually based on clinical association, as there are no constant abnormalities in the special tests; however, in some disorders the tourniquet test is positive and/or the bleeding time is prolonged.

In some hemorrhagic disorders, it is not uncommon for more than one component of the hemostatic mechanism to be involved, e.g. cirrhosis of the liver, in which there may be 'hypoprothrombinemia' from liver damage and thrombocytopenia due to hypersplenism.

WHAT IS CAUSE OF THE HEMORRHAGIC DISORDER?

The cause is determined from a consideration of the history and examination, and certain special tests.

The history of bleeding in relation to past trauma is of particular help in determining the cause of the bleeding. A long history of abnormal bleeding, particularly when it commences in childhood, is strong evidence that the disorder is congenital. However, acquired disorders may persist for years before the diagnosis is made. On taking the history, a record should be made of the various traumatic incidents that have been experienced and of abnormal bleeding if this has occurred with any of them. Severe tests of hemostatic efficiency are imposed, particularly by tooth extraction and tonsillectomy, and in females by the menstrual cycle. In mild hemorrhagic disorders.

CHAPTER

18

Thrombosis: Clinical Features and Management

Thrombosis and atherosclerotic vascular disease are major causes of morbidity and mortality, and they increase in incidence with advancing years. There are many contributing factors and predisposing conditions, and the mechanism of thrombus formation itself is complex and only partially understood. Continued investigation is required to provide a rational basis for prevention and treatment. This section examines some of the mechanisms involved, the clinical syndromes resulting from thrombosis, and to outline current approaches to their management.

DEFINITION OF THROMBOSIS

A thrombus may be defined as a mass of aggregated platelets, adherent to the vessel wall and immobilized with fibrin. There is a variable content of red cells and entrapped leukocytes, and the proportions and arrangement of the various components depend on local and general conditions.

TYPES OF THROMBUS

The size and constitution of a thrombus depend on general factors (components of the blood), local factors (the blood flow and vessel wall) and the site where thrombus formation occurs, i.e. whether it is within the arterial or venous circulation.

VENOUS THROMBOSIS

It is more common when there is sluggish flow or stasis, and endothelial changes are rarely the main causative factor. Such a thrombus is usually composed of abundant fibrin and many red cells. It generally resembles the appearance of clots formed in glass tubes, although the leading, or most proximal, portion often contains prominent platelet masses and is paler than the distal coagulum of red cells and fibrin.

ARTERIAL THROMBOSIS

It frequently occurs around the orifices of branches and at bifurcations. It is in these areas, where turbulence and sheer stresses are greatest, that endothelial injury and atheromatous changes are most marked, and platelet aggregates are readily formed. Such platelet aggregates may adhere locally and may progressively increase in size as more platelets adhere to the surface. Some coagulation and fibrin formation may occur, and limited red cell entrapment follows. An arterial thrombus thus formed has a pale appearance due to the predominance of platelets.

EFFECTS OF THROMBOSIS

Thrombosis may produce both local and distant effects. The local effects depend on the site and the degree of vascular occlusion, and the remote effects are due to embolic phenomena or to the release of vasoactive substances from the evolving thrombus into the passing stream of blood.

Venous thrombosis may result in complete obstruction of major channels such as the popliteal, femoral, or iliac veins with distal edema, and in exceptional circumstances (such as the mesenteric circulation) may cause tissue infarction. Detachment and embolization of various thrombi may produce obstruction within the pulmonary arterial system (pulmonary emboli).

Local occlusion at the site of initial thrombus formation in arteries is nearly always associated with intimal disease or microscopic damage. Such occlusion usually produces marked ischemic damage and organ dysfunction. The platelet aggregates within an arterial thrombus are often unstable and readily break up, releasing platelet masses into the circulation. Many such aggregates may disperse spontaneously, with return of the platelets to the general circulation. Other platelet masses may produce transient or permanent obstruction in distant small vessels, as is well recognized in the retinal and cerebral circulations.

There is a strong possibility that vascular spasm (for example, of the coronary arteries) may be caused by thromboxane A_2, serotonin, or other vasoactive substances released as a consequence of platelet activation. Such spasm may cause ischemic symptoms, particularly if the circulation is already compromised by proximal atheroma.

ETIOLOGY OF THROMBOSIS

The etiology of thrombosis is a complex subject, and in most cases is multifactorial. Many associations of clinical thrombosis have, as yet, ill-defined etiological relationships. Although there are differences between the factors predisposing to arterial and venous thrombosis, considerable areas of overlap exist. Arterial thrombosis and vascular disease are considered first, then the factors predisposing to venous thrombosis, and, finally, the condition of disseminated intravascular coagulation and fibrinolysis.

VASCULAR DISEASE AND ARTERIAL THROMBOSIS

There is a close relationship between thrombosis and the development of atherosclerotic vascular disease. There are at least three ways in which blood components may contribute to the development of atherosclerosis and its complications:

a. By hemodynamic factors and platelet-leukocyte interaction with the vessel wall which may lead to endothelial injury and consequent smooth muscle migration and proliferation.
b. By the formation of persistent mural thrombi which are organized and incorporated into the subendothelium, potentiating vessel wall damage.
c. By formation of thrombi in association with advanced atherosclerosis. Vessel wall disease develops through the phases of intimal thickening, medial muscle hypertrophy, lipid accumulation, and later calcification. These result in rigidity, lumen reduction, and disturbed flow, and provide the setting for platelet adherence and thrombus formation.

The early lesions of atherosclerosis, particularly the migration and proliferation of smooth muscle cells, are probably mediated by growth factors released either by platelets or by macrophages attracted to sites of vascular injury. Related mitogens may be released

by the vessel wall cells themselves, endothelium and smooth muscle, also as a response to injury.

A number of clinical and laboratory parameters have been identified associated with the development of atherosclerotic vascular disease. Modification of many of these factors may reduce the likelihood of progression of vascular disease and the development of its complications, although it has been surprisingly difficult to prove the value of alterations in dietary habits. Mortality statistics, despite their acknowledged deficiencies, suggest a substantial reduction in deaths from ischemic heart disease over the past 20 years in a number of western countries. This is likely to be due, in part, to modifications of the presumed risk factors, particularly reduction in weight, dietary saturated fats, and cigarette smoking, and the earlier treatment of moderate hypertension. Some of the more important 'risk' factors are now briefly mentioned in Fig. 18.1.

Hyperlipidemia

A high level of plasma cholesterol and triglycerides have both been shown to lead to atherosclerotic change in arteries in both experimental animals and humans. Platelets are also susceptible to lipids in their environment and are aggregated by fatty acids, and it is possible that platelet aggregates may form more readily when the lipid pattern of plasma is altered. Hyperlipidemia and obesity are also associated with decreased plasma fibrinolytic activity, possibly with enhanced coagulation, both of which might aggravate the thrombotic tendency. A high incidence of premature peripheral vascular disease and myocardial infarction is found in persons with hyperlipoproteinemia. More recently, it has been suggested that the detrimental effect is associated with the low-density lipoprotein cholesterol fraction. An increased proportion of high-density lipoprotein cholesterol is protective against the development of coronary disease in epidemiological studies.

Diabetes Mellitus

Hyperlipidemia is not uncommon in diabetes, and there is some evidence to suggest enhanced coagulation and increased platelet responsiveness. More striking, however, are functional abnormalities of endothelium that may result in increased tendency to platelet overreactivity.

Smoking

The increased mortality from ischemic heart disease, and morbidity from peripheral vascular disease and cerebrovascular disease, associated with cigarette smoking is well-documented. The mechanisms underlying the toxicity of tobacco smoke are poorly

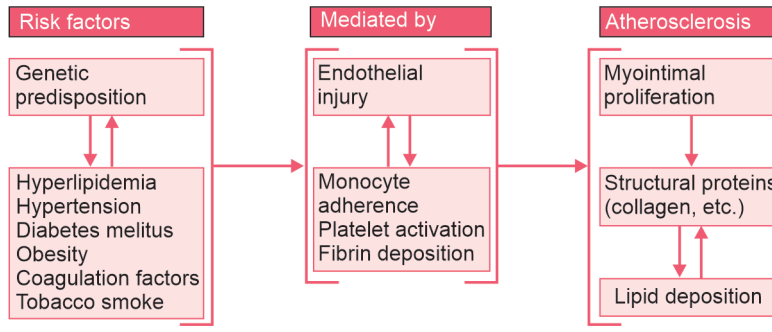


Fig. 18.1: Relationship between clinical risk, hemostatic components, and the development of atherosclerosis

understood. There is some evidence for increased platelet aggregability due to smoking. Both animal experiments and in vitro studies point to toxicity of tobacco products on vascular endothelium. Carbon monoxide and nicotine have been implicated, but the evidence is not overwhelming.

Exercise and Body Build

The role of exercise and leanness of body as factors that might reduce the incidence of thrombosis have been widely debated. There have been retrospective studies suggesting that myocardial ischemia is less common in those who exercise regularly, but the relationship remains to be confirmed by properly conducted prospective studies.

Hypertension

Hypertension may produce endothelial injury, and focal increases in endothelial cell replication have been shown in hypertensive animals. At the clinical level, both prospective studies and the benefit of treatment of mild to moderate hypertension suggest that hypertension is not important risk factor.

Hypercoagulability

Research has shown that hemostatic variables, studied prospectively, are important in the risk of subsequent cardiovascular death, principally from ischemic heart disease. High levels of factor VIIc and fibrinogen are associated with a high incidence of coronary artery disease. Other measurable hemostatic components important in the development of cardiovascular events are platelet aggregability and antithrombin III. Lowered antithrombin III levels are associated with estrogen administration and an increase in cardiovascular events.

Inherited, Racial, and Dietary Factors

These factors must also contribute as the incidence of thrombotic disease is not uniform in different countries. Changes in dietary habits in immigrant racial groups have also been associated with development of thrombotic disorders, suggesting the importance of dietary and environmental factors. Molecular techniques may in the future enable screening for specific genetic characteristics associated with vascular disease, e.g. identifying the genes for apolipoproteins which particularly predispose to lipid accumulation.

CONDITIONS ASSOCIATED WITH VENOUS THROMBOSIS (Table 18.1)

Congenital

Antithrombin III Deficiency

This is a rare disorder in which there is reduced activity of the natural anticoagulant (inhibitor) against activated factor X (factor Xa) and thrombin. Deficiency is inherited as an autosomal dominant and is associated with a high incidence of venous thrombosis and pulmonary embolism, usually presenting in the second or third decade. Plasma concentrations of 50% of normal are sufficient to predispose to thrombosis. In most cases, the plasma antithrombin III concentration is reduced, but families with dysfunctional and pathological antithrombin III molecules have been described.

Table 18.1: Conditions associated with venous thrombosis

- Advanced age
- Obesity
- Surgery
- Trauma
- Prolonged recumbency
- Pregnancy and puerperium
- Oral contraceptives*
- Myeloproliferative syndromes*
  - Polycythemia vera
  - Essential thrombocythemia
- Malignancy
- Varicose veins infection
- Previous venous thrombosis
- Inflammatory diseases
- Paroxysmal nocturnal hemoglobinuria*
- 'Lupus' anticoagulant (antiphospholipid antibody syndrome)*
- Conditions associated with disseminated intravascular coagulation*
- Hereditary coagulation protein abnormalities

* Predispose to arterial thrombosis as well.

Protein C Deficiency

Recurrent venous thromboembolic disease is associated with reduced plasma levels of the vitamin K-dependent factor, protein C in a small number of heterozygotes in affected families reported since 1981. Inheritance is autosomal. Homozygous infants are stillborn or suffer perinatal purpura fulminans. A similar deficiency associated with thrombosis has been reported with protein S.

Plasminogen Abnormalities

Rare, functionally deficient plasminogen molecules have been described in association with reduced fibrinolytic activity and a tendency to venous thrombosis.

Dysfibrinogenemia

Occasional cases of increased thrombotic tendency have been attributed to functionally abnormal fibrinogen.

Acquired

Surgery and trauma and prolonged recumbency for any reason are associated with a high incidence of venous thrombosis. Surgery and trauma are characterized by a state of relative hypercoagulability of the blood. When sensitive techniques such as venegraphy and 125 I fibrinogen scanning are used, many small areas of thrombosis are found; as many as 50% may not be noticed clinically. For the same reason (immobilization), long haul flights also predispose to thromboembolic phenomena. The diagnosis has been simplified with the advent of color Doppler studies.

During pregnancy, and particularly in the *puerperium,* there is also a state of relative hypercoagulability, which may result from increased estrogen levels in the blood. The activity levels of factors VII, VIII, and X are increased, and there is also a tendency for other clotting factors, particularly fibrinogen, to be elevated and for fibrinolytic activity to be decreased. During labor, tissue damage leads to activation of coagulation and readily

detectable alterations in a number of factor activities, platelet count, and fibrinolysis, consistent with a state of compensated intravascular coagulation. In multiple pregnancies, or when labour is prolonged, these changes are more marked and may lead to over thrombosis in the puerperium. Amniotic fluid embolism, associated with dissemination of fetal material, may also lead to intravascular coagulation.

Usage of oral contraceptive medication is established as a significant association with thromboembolic disease. The incidence of thromboembolism in women taking oral contraceptives has been shown to be nine times as compared to normal population. This includes calf vein thrombosis, cerebral and mesenteric thrombosis, and, in older women, myocardial infarction, although the evidence for the latter is suggestive rather than conclusive. Increased levels of coagulation factors VII and X, and decreased antithrombin III, as well as altered platelet activity, occur during medication with oral contraceptives. Some changes have also been found in factor VIII levels and fibrinolytic activity. The incidence of thrombosis is reduced by the use of preparations containing low dosage of estrogens. It is generally recommended that the oral contraceptives should be discontinued 2–3 months before elective surgery to diminish the risk of postoperative venous thrombosis.

FACTORS PREDISPOSING TO BOTH ARTERIAL AND VENOUS THROMBOSIS

Age and Sex

There is an increasing incidence of both arterial and venous thrombosis in older individuals. Male sex is associated with a higher incidence of vascular disease and thrombotic incidents.

Thrombocytosis

In association with *myeloproliferative disorders* predisposes to thrombosis, which may be venous or arterial. This is the case particularly in polycythemia vera, when erythrocytosis and hyperviscosity (most severe when the PCV is in excess of 0.60) greatly increase the risk of the thrombosis. Myocardial, cerebral, digital, or gastrointestinal infarction may all occur under these conditions.

Essential Thrombocythemia

In essential thrombocythemia bleeding and thrombosis may be present at the same or different times in the one patient. In the thrombocytosis of chronic granulocytic leukemia.

Thrombosis may occur, especially when the total white blood cell count is elevated about 400×10^9/L and the effective packed cell volume over 0.60, but this is much less frequent complication than with polycythemia.

Thrombocytosis

Thrombocytosis may predispose to thrombosis, especially when the platelet count is elevated above 800×10^9/L. The risk of thrombosis is increased in patients with vascular disease and is enhanced by immobilization. The latter situation arises most frequently after splenectomy, when special care must be taken to ensure early ambulation.

Malignancies

Many malignancies may also lead to thrombosis. This is a well-recognized complication of some abdominal malignancies such as carcinoma of the pancreas and some mucin secreting adenocarcinomas. These may give rise to recumbency-type venous thrombosis or more

superficial thrombophlebitis. Extensive investigation has shown some manifestations of intravascular coagulation in many patients with cancer. Occasionally, a state of overt chronic disseminated intravascular coagulation may develop. This may be responsive to heparin and to treatment directed against the tumor, if localized. In some cases, the mechanism of thrombosis is the release of clot-promoting thromboplastic materials from the tumor. There is some direct evidence to support this possibility, in that some malignant tissues can be shown *in vitro* to contain excessive clot-promoting substances.

Inflammatory Mediators

It is now understood that inflammatory mediators such as interleukin-I and tumor necrosis factor produce a number of changes in vascular endothelium that are procoagulant in nature. These changes include the production of tissue factor, release of von Willebrand factor and platelet activating factor, and the secretion of plasminogen activator inhibitor. As the endothelium may provide membrane-associated cofactors for coagulation reactions, all the machinery for local fibrin formation is available at sites of tumor metastasis.

Infections

It may be associated with an increase in coagulability, and similar mechanisms may pertain. This is seen in some cases of malaria, but it is especially a feature of septicemia due to gram-negative organisms. The endotoxins produced are probably responsible for initiating coagulation, possibly because of effects on platelets and endothelium. Associated tissue damage and hypoxia, especially if there is circulatory failure, also contribute. Superficial venous thrombosis may occur, as may disseminated intravascular coagulation. A bleeding tendency results, and may be an important factor in determining the outcome. The thrombocytopenia often observed under these conditions may also result in part from bone marrow suppression due to infection and endotoxinemia.

Homocystinuria

This metabolic disorder is associated with premature thromboembolic complications, both venous and arterial. Abnormally high concentrations of plasma homocystine are believed to injure the endothelium, but the experimental evidence is far from secure. It has also been suggested that heterozygotes may have an increase in the incidence of arterial disease.

'Lupus' Anticoagulant

Circulating IgG or IgM antibodies directed against components of phospholipid are detected in over of 15% of patients with *systemic lupus erythematosus,* in patients with other connective diseases, and in association with a wide variety of other disorders. The antibody is termed anticoagulant because it causes prolongation of the activated partial *thromboplastin time* (APTT), and sometimes the *prothrombin time* (PT), which is not corrected by the addition of an equal volume of normal plasma. Both, the dilute Russell viper venom test and the kaolin clotting time are sensitive tests to confirm the diagnosis.

The clinical expression is paradoxically a striking tendency to thrombosis and to spontaneous abortion (presumably due to placental insufficiency), and a bleeding tendency, if it occurs, is very uncommon. The thrombosis may be a result of vascular endothelial cell damage, reduced prostacyclin production, or interaction with platelet-activating factor, but is presently not explained.

Lupus anticoagulation and related *antiphospholipid antibodies* can be detected by a recently introduced solid phase immunoassay using cardiolipin as the antigen. Cardiolipin is representative of the negatively charged phospholipids, and others, such as phosphatidylserine, cross react in these assays. Some patients with thrombosis have detectable antiphospholipid antibodies without lupus anticoagulant, and *vice versa*, but in many, both tests are positive and it is likely that the assays reflect variability in a family of closely related antibodies.

Corticosteroids may temporarily abolish the lupus anticoagulant and reduce the titer of antiphospholipid antibodies, but if there is a history of thromboembolic disease, long-term antithrombotic treatment is indicated either with warfarin (particularly with venous disease) or aspirin (in the case of arterial disease). Recurrent abortion with antiphospholipid antibodies has been successfully treated with a combination of prednisolone and aspirin.

Paroxysmal Nocturnal Hemoglobinuria (PNH)

Major thrombosis may occur at varying sites as a result of intravascular coagulation associated with severe hemolysis that occurs in the more classic form of this disease. Anticoagulant treatment with vitamin K antagonists has been advocated in PNH.

Disseminated Intravascular Coagulation

Both aterial and venous thrombosis may occur in this condition. Its occurrence has been noted above in association with pregnancy, carcinoma, infection, and PNH, and disseminated intravascular coagulation with thrombotic complications may occur with incompatible blood transfusion and thrombotic thrombocytopenic purpura.

CLINICAL SYNDROMES OF THROMBOSIS

ARTERIAL THROMBOSIS

While thrombosis may complicate a variety of diseases and metabolic alterations, there are a number of well-recognized syndromes that deserve consideration. Many of these are recongized as disease entities, although in some cases the etiology is obscure or complex; however, all result from some disturbance in the balance of hemostasis leading to thrombosis and vascular occlusion (Table 18.2).

Table 18.2: Arterial thrombotic syndromes

- Myocardial ischemia and infarction
- Occlusive cerebrovascular disease and transient ischemia
- Peripheral arterial occlusive disease
- Homograft rejection

Disorders of uncertain or varied etiology

- Hemolytic uraemic syndrome
- Purpura fulminans
- Thrombotic thrombocytopenic purpura
- Disseminated intravascular coagulation

MYOCARDIAL ISCHEMIA AND INFARCTION

Myocardial infarction (MI) refer to irreversible ischemic muscle damage resulting from impaired blood flow in the coronary arterial system. This is usually associated with atherosclerotic changes in these arteries, sufficient to cause narrowing of the lumen, and

a thrombus may be found either occluding the lumen or adhering to the vessel wall. However, in some instances, no thrombus can be detected, and full patency is present despite the existence of atheromatous change.

Detection of thrombus at autopsy is related to the care with which it is sought, and it is probable that at the time of onset of a particular ischemic episode thrombus formation always occurs. Surgical findings and early coronary angiography have confirmed that thrombosis occurs almost invariably with, and is presumably the cause of, full thickness myocardial infarction. However, if the examination of postmortem specimens is undertaken many hours after the onset of symptoms in fatal episodes, thrombi may have been dislodged or lysed in the natural process of clot dissolution and repair.

Coronary arterial atheroma is the cause of myocardial ischemia, and may not give rise to infarction. The role of thrombosis in minor ischemic episodes is difficult to determine. It is probable that platelet aggregation frequently occurs around the orifice of diseased coronary arteries, since this is a region of particular turbulence. Even though such aggregates may be transient and not associated with fibrin formation, for example by including vascular spasm. In such a situation—unstable angina—there is direct evidence that aspirin, an inhibitor of platelet aggregation, reduces the incidence of myocardial infarction. The accumulation of clinical trial results also suggests that aspirin reduces both the incidence of nonfatal reinfarction and the subsequent mortality after myocardial infarction. There is similarly convincing evidence to favor the use of anti-coagulants, at least during hospitalization after acute myocardial infarction, to prevent venous thromboembolism.

OCCLUSIVE CEREBROVASCULAR DISEASE AND TRANSIENT ISCHEMIC ATTACKS

The carotid arteries, particularly at their bifurcation, are important sites of atheroma formation. Intimal thickening and partial occlusion are commonly observed with advancing years. Similar changes may be encountered in intracerebral arteries, and it is only the presence of a rich collateral supply that spares many areas from the effects of ischemia. Thrombotic episodes causing occlusion may produce a wide range of symptoms, from transient weakness to fully developed stroke resulting from extensive cerebral damage. It is common to detect thrombus after a major episode of cerebral ischemia. Thrombotic occlusion may involve the carotid system, or may be found in smaller vessels such as the middle cerebral or the vertebrobasilar system. In some cases, major occlusion does not occur, and symptoms arise because of embolism of platelet aggregates or small thrombi from the surface of plaques of atheroma in the carotid vessels. These emboli may produce permanent damage, such as blindness from central retinal artery occlusion, or hemiparesis due to occlusion of the middle cerebral artery or its branches.

Alternatively, only transient ischemia may occur, giving rise to reversible episodes known as transient ischemic attacks. Such emboli have been seen by direct retinal observation, strongly supporting their role in these episodes. It is also well-recognized that transient ischemia may progress to major strokes, and this has led to vigorous attempts to deal with the disorder by both medical means and by surgery, chiefly carotid endarterectomy. This operation often has beneficial results in selected patients.

Knowledge so far also suggests that inhibitors of platelet aggregation, in particular aspirin many patients and decrease the incidence of transient ischemic episodes. Vitamin K antagonists have been shown to be at least as effective, but carry a greater risk of bleeding complications.

PERIPHERAL ARTERIAL OCCLUSIVE DISEASE

The aorta and its major branches are almost always the sites of atheromatous change with advancing years. A degree of vessel narrowing and rigidity is common, but actual occlusion due to thrombosis is confined to branches such as the mesenteric, renal, and iliac arteries, and embolic blockage may occur with local ischemia or infarction in more distal vessels. Any of these systems may be blocked by emboli arising more proximally, either from more central areas of atherosclerosis, or from mural thrombus formation in the left ventricle after myocardial infarction.

Claudication or ischemic limb pain usually results from extensive disease of the large arteries and their major branches. These same vessels are the ones usually involved with major occlusive emboli. However, digital thrombosis with terminal ischemia may occur in the absence of major vessel disease. Small arterial occlusion of this type is a feature of diabetic atherosclerosis, and is also seen with inflammatory vasculitis, as in scleroderma and other collagen diseases. It may also occur due to hyperviscosity and sluggish flow, as in cold agglutinin disease (with hyperglobulinemia and red cell agglutination) and in polycythemia. Small vessel occlusion and digital gangrene may also complicate cryoglobulin syndromes. In these cases, preexisting atheroma in small vessels may predispose to occlusion, but actual thrombosis is not an essential accompaniment.

Thrombosis within the heart may be a source of peripheral emboli. This may occur with the flow disturbances associated with mitral stenosis and atrial fibrillation. Thrombus formation in the atrium is not uncommon, and may result in embolization when sinus rhythm is restored. Similarly, thrombus formation is a component of the valve deposits occurring in subacute bacterial endocarditis. Prosthetic heart valves are also the site of thrombus formation, and this occurs with such regularity that continuous treatment with oral anticoagulants is required. Thrombus formation within the ventricles may follow myocardial infarction and result in embolization in the periphery or in the pulmonary circulation.

Homograft rejection is associated with thrombosis, well described in the experimental animal and in the transplanted kidney in humans. The immune reaction to foreign tissue mediated by lymphocytes is marked by perivascular inflammation with mononuclear cells predominating, and by intravascular platelet aggregates and thrombus formation. The resultant ischemia and tissue damage result in rejection. The mechanism of thrombosis under these circumstances is not known. Tissue damage resulting from the action of lymphocytes may be the first event, but antigen-antibody complexes may also be present and contribute to the platelet release reaction and aggregation. Since prevention of rejection and control of threatened rejection depend on immunosuppressive agents and not antiplatelet drugs or anticoagulants, thrombosis must be a secondary event. However, there is evidence that antithrombotic drugs and anticoagulants may prevent the renal vascular lesions associated with graft rejection, so it is likely that the process does depend to certain degree on thrombus formation.

Arteriovenous shunts used for chronic hemodialysis suffer from a liability to thrombotic occlusion. This may lead to repeated revision and surgical correction, and may maneuver that reduces this requirement in beneficial. The details appear below, but combinations of vitamin K antagonists, heparin during perfusion, and platelet aggregation inhibitors have reduced the incidence of shunt blockage.

VENOUS THROMBOSIS

Superficial thrombophlebitis is a condition in which tender swellings develop on superficial veins. It is often associated with prolonged infusions with indwelling catheters and

the injection of irritant chemicals. Local thrombosis is an accompaniment, but may not extend to deeper veins. Localized tender areas around superficial veins may be difficult to distinguish from thrombophlebitis, and are sometimes called superficial vasculitis. An element of thrombosis in superficial vessels may be involved in this condition, which may also accompany more diffuse small vessel disease in some of the connective tissue disorders. A similar superficial vasculitis may have no discernible underlying basis, but also occurs in association with hyperglobulinemia of polyclonal type and purpura chiefly affecting females, initially described by Waldenstrom.

Deep venous thrombosis, involving the veins of the soleal system in the calf, characteristically presents with a tender, swollen calf, ankle edema, distended superficial veins in the foot, and mild fever. However, in as many as half the cases if may be asymptomatic and have no superficial accompaniment. More sensitive diagnostic procedures such as ^{125}I fibrinogen scanning and venography may then be required. In addition, a deep venous thrombosis may extend proximally to the popliteal, femoral, and iliac veins, or may arise primarily in pelvic veins. Sometimes, major ileofemoral venous thrombosis may give rise to arterial obstruction and the clinical picture known as 'phlegmasia caerulea dolens'. The limb becomes swollen, discoloured and purple with absent pulses, and gangrene may develop. The condition thus has a grave prognosis.

When occurring postsurgery, the clinical onset of lower limb venous thrombosis tends to be delayed for several days, and may occur as late as 10–14 days after operation, even though the initial thrombus formation probably occurred during operation. It is associated with a tendency to increased coagulation, and sometimes elevation of some coagulation factor activities and an increased platelet count. In some studies, platelet aggregability appeared to be increased, but it is not certain that these alterations in coagulation and platelet numbers and function are directly related. There may be other factors of equal importance, such as age, associated disease, and the nature of the predisposing trauma or surgery.

Thrombosis in iliac, pelvic, or peripheral veins results in pulmonary embolism in a significant proportion of patients. There may be little or no clinical evidence of quite large venous thrombi capable of producing a major pulmonary embolus. Indeed, when the triad of local pain, tenderness, and edema is present, the diagnostic techniques of venography and ^{125}I fibrinogen scan confirm the diagnosis in 80–90% of cases. Now color Doppler has made the diagnosis much easier. However, the sensitivity of clinical signs is low, and at least 50% of cases are overlooked.

These evidences, together with the serious outcome of many such emboli, have led to increasing efforts to define patients at risk and to offer preventive treatment. According to established criteria, a major pulmonary embolus may be defined as one that produces impairment or abolition of the blood flow to more than one-third of both lungs or two-thirds of one lung. Such acute embolization may be immediately fatal, and almost always produces symptoms; it is detectable by pulmonary angiography and perfusion lung scanning with radioisotopes, and produces changes in cardiopulmonary flow parameters. A similar degree of functional impairment may result from repeated embolization from a chronic peripheral source, but usually without the dramatic picture of acute embolization. Recovery from major pulmonary embolism is followed by gradual resolution of the perfusion defects as fibrinolysis and repair take place. Often some months must elapse before these defects resolve, and indeed they may persist for long periods after full symptomatic recovery and the apparent return of lung function tests to normal.

MICROCIRCULATION THROMBOSIS

Hemolytic uremic syndrome: This is a severe, acute illness of early childhood. It is characterized by the rapid onset of severe anemia and thrombocytopenia, with renal failure and evidence of small vessel thrombosis, and in some cases intravascular coagulation.

Thrombotic thrombocytopenic purpura is a severe, uncommon illness of adults, resembling somewhat the hemolytic uremic syndrome of childhood. In addition to thrombocytopenic bleeding, hemolytic includes cerebral involvement, often producing coma.

Disseminated intravascular coagulation (DIC) and pathological fibrinolysis are considered elsewhere.

INVESTIGATIONS OF THROMBOTIC DISORDERS (Table 18.3)

The diagnosis and localization of established vascular occlusion is beyond the scope of this chapter, and the reader is referred to texts devoted to thrombosis or vascular disease. Clinicians may, however, avail themselves of hematological investigations for: (a) evidence of established thrombosis; and (b) the diagnosis of a predisposition to thrombosis, be it congenital or acquired.

On the whole, blood tests for established thrombosis have proven disappointing because of lack of specificity. Fibrin formation and platelet activation accompany the common processes of inflammation, repair following surgery or trauma, and tumor growth and metastasis. If evidence of thrombosis is detected in the blood, all such conditions must be excluded before much weight can be put on such findings.

The transient nature of the thrombotic process and the products of thrombosis in the blood is a further impediment to assays designed to diagnose thrombosis. Finally, technical difficulties in both preparation of plasma samples and the execution of assays for products of thrombosis prevent their widespread acceptance. Thus, much work has gone into developing specific assays for products in plasma of thrombin action (fibrinopeptide A and

Table 18.3: Screening tests for predisposition to thrombosis

Must consider	*Causes*
Blood count and film	Polycythemia, thrombocytosis, PNH
Activated partial thromboplastin time	Lupus anticoagulant, antiphospholipid antibody and DIC
Prothrombin time	
Thrombin clotting time	Dysfibrinogenemia
Anticardiolipin antibody	Antiphospholipid antibody
Antithrombin III assay	Antithrombin III deficiency
Protein C assay	Protein C deficiency
Protein S assay	Protein S deficiency
Less certain clinical significance	
Euglobulin fibrin clot lysis before and after venous stasis	Abnormalities of plasminogen activator release, or activator inhibitor excess
Plasminogen activator inhibitor assay	
Platelet aggregation to standardized agonists	Abnormal platelet sensitivity
Very rare conditions	
Assays for rare abnormalities of these molecules	Plasminogen, heparin cofactor II, fibrinogen studies

thrombin–antithrombin complexes), platelet activation and release (β thromboglobulin and platelet factor-4), and plasmin action (fibrin/fibrinogen degradation products). Only the tests for fibrin degradation products, either those that nonspecifically detect products from fibrinogen or fibrin or those specific for cross-linked fibrin degradation are in widespread use, mainly for diagnosis of disseminated intravascular coagulation. As indicated above, the tests have not been found to be sufficiently specific in, for example, the diagnosis of deep venous thrombosis.

In the case of unexplained thromboembolism in patients of less than 40–45 years of age, or recurrent thromboembolism with a suggestive family history, screening for an inherited or an acquired predisposition is indicated. In general terms, functional assays are preferable, although for convenience immunoassays are commonly employed.

MANAGEMENT OF THROMBOTIC DISORDERS

With greater than ever understanding of the function of platelets and of the processes of blood coagulation and fibrinolysis, the management of thrombotic disorders has become a complex matter. There are now available a large number of compounds active against platelet aggregation and the release reaction. The use of heparin has undergone extensive reevaluation since the discovery of the central importance of anti-Xa (antithrombin III) as heparin cofactor. There has been no significant change in the use of vitamin K antagonists, but there is a tendency to use them in combination with antiplatelet agents, dipyridamole in particular, in some situations. Thrombolytic therapy with streptokinase and urokinase has undergone continuing investigation, and the development of more fibrin-selective thrombolytic agents is well advanced. Tissue-type plasminogen activator produced by tissue culture and more recently by recombinant DNA techniques will very likely replace the other plasminogen activators in the future. There is little doubt of their benefit in the treatment of major pulmonary embolism, but cost considerations have tended to restrict their use. The treatment of early acute myocardial infarction with thrombolytic agents has become routine following evidence from early coronary angiography and intracoronary administration of drugs and subsequent large-scale studies of high-dose intravenous administration. The recognition of the involvement of rheological changes and the effects of turbulence and high viscosity have also led to the use of materials such as dextrans which may enhance flow and decrease viscosity (Table 18.4).

Table 18.4: Agents available for the treatment of thrombotic disorders

Inhibitors of platelet function Natural: Prostacyclin, prostaglandin E_1 (PGE_1) Chemical: Aspirin, sulphinpyrazone, dipyridamole, hydroxychloroquine, ticlopidine, clopidogrel
Anticoagulants Heparin in low and high doses Low molecular weight heparin and heparinoids vitamin K antagonists
Thrombolytic agents Streptokinase, urokinase and tissue plasminogen activator (t-PA)
Perfusion enhancement Dextran (antiplatelet effect)
Enhancement of fibrinolysis Phenformin and ethyloestrenol Stanozolol

In spite of all these developments, there are persisting areas of uncertainty. There is a continuing need for carefully controlled trials of the use of several of the available agents. It would be helpful if combinations could be avoided before it is shown that there is a real benefit to be derived from them.

Diagnostic criteria must be carefully defined, especially where the treatment commitment is relatively long-term, as in the case of peripheral vein thrombosis and cerebral ischemic episodes, or complex and expensive as in the case of thrombolytic agents. It is the aim in this section to present guidelines for the treatment of various thrombotic states.

INHIBITORS OF PLATELET FUNCTION

Platelet aggregation, as assessed *in vitro*, occurs in two phases. The first is reversible and is not associated with a release reaction. The second, an irreversible phase of aggregation, occurs with release of platelet adenosine diphosphate (ADP) and other constituents. A number of natural compounds, including adenosine and prostaglandin E_1 (PGE_1), are potent inhibitors of ADP-induced aggregation, but produce unacceptable side effects, preventing therapeutic trials. Synthetic prostacyclin, however, has had limited clinical trial, and efforts to produce a more stable analog may result in a potent antithrombotic drug.

A group of nonsteroidal anti-inflammatory agents, including acetylsalicylic acid and phenylbutazone, and to a lesser extent the uricosuric agent sulphinpyrazone, inhibit platelet cyclooxygenase. They thus interfere with the release action and secondary aggregation induced by ADP and noradrenaline. They do not inhibit ADP-induced primary aggregation. Aspirin ingestion results in a significant prolongation of the bleeding time and a detectable effect on platelet aggregation persists for a number of days due to acetylation and permanent inhibition of platelet cyclooxygenase. Any cyclooxygenase inhibitor has the inherent disadvantage of inhibiting this enzyme in the vascular endothelium and thus reducing vascular prostacyclin production. This drawback may be more apparent than real, as endothelial enzymes are less affected and, in addition, are being renewed constantly. This is not so in the platelet, which is devoid of synthetic apparatus. Daily doses of 50–100 mg aspirin may provide the desired platelet inhibition without reducing endothelial prostacyclin. The clinical efficacy of such dosage, however, has been demonstrated only in limited studies, in the prevention of renal dialysis shunt and coronary artery bypass graft occlusions.

Dipyridamole, by inhibiting platelet phosphodiesterase, results in accumulation of cyclic adenosine monophosphate (cAMP) with a demonstrable effect *in vitro* on platelet aggregation. Ticlopidine, a new drug with unique antipatelet activity, is showing promise in clinical trials.

A number of large clinical trials have been conducted, mainly in arterial thrombotic disease, and the emerging picture is that aspirin is the only definitely useful agent. In venous thrombosis, antiplatelet agents probably have a very limited therapeutic potential.

ARTERIAL THROMBOSIS

In the past, it was noted that the incidence of fatal myocardial infarction was less in patients with arthritis or other conditions treated by prolonged aspirin ingestion than in an age-matched population. Whether the two were connected as cause and effect could be established only by large-scale prospective studies, and these have been slow to accumulate convincing answers.

The principal agents which have been subjected to adequate clinical trial are aspirin, dipyridamole, sulphinepyrazone, and hydroxychloroquine. *Dipyridamole* was found to

reduce the incidence of experimental thrombosis, and clinical trial suggested that it had an important influence on the incidence of postoperative thromboemboli after cardiac valve prosthetic surgery in combination with vitamin K antagonists. Dipyridamole in combination with aspirin was found to prolong the shortened platelet survival seen in patients with cardiac valve prostheses. The incidence of transient cerebral ischemic attacks was reduced by dipyridamole in combination with aspirin, although the most convincing demonstration of a therapeutic effect of antiplatelet therapy in this group of patients was achieved by aspirin alone. Overall, the clinical evidence to support the use of dipyridamole as an antithrombotic agent is not convincing.

Many well-conducted trials, which suggest benefit from the use of sulfinpyrazone, aspirin, and hydroxychloroquine in the prevention of shunt thrombosis and blockage of renal dialysis membranes. There are also indications of benefit in elderly patients prone to thrombotic disorders. A series of trials of these drugs given to patients after acute myocardial infarction suggest that a modest reduction in mortality in the first few months after the event may be obtained by the administration of sulfinpyrazone but more convincingly by aspirin. In addition, the rate of nonfatal reinfarction over the first 12 months is reduced by some 15–20%. Aspirin at a dose of 325 mg daily reduces the mortality and infarction rate in patients with unstable angina and also substantially reduces the occlusion rate of CABG.

ANTICOAGULANTS

Heparin

This naturally occurring anticoagulant is a mucopolysaccharide which is highly charged and has the ability to bind to proteins. The anticoagulant action of heparin requires the presence of cofactors, the major component of which has been identified as antithrombin III and the lesser, heparin cofactor II. The major site of action is probably against factor-Xa, and the mechanism would appear to be marked potentiation of antithrombin III (also known as anti-Xa). Factor-Xa has a potent enzymatic action on prothrombin in the presence of factor-V, phospholipid, and calcium ions, and the catalytic effect of factor-Xa is to produce quite large amounts of thrombin. The kinetics of these reactions are such that small amounts of heparin in the presence of antithrombin III prevent the formation of large amounts of thrombin. Inhibition of factor-Xa by antithrombin III is nearly instantaneous in the presence of heparin, whereas the reaction is much slower in its absence. Once thrombin is formed, however, larger quantities of heparin are required to exert an anticoagulant effect. Heparin has a minor action in inhibiting the activation of factor-IX by factor-XIa, and the effect of factor-IXa.

The major indications for heparin are:

- Postoperative or recumbency stasis thrombosis
- Prophylaxis of deep venous thrombosis with recent myocardial infarction or other predisposing condition
- Treatment of established deep venous thrombosis with or without pulmonary embolism
- Maintenance of anticoagulation in extracorporeal circulations
- Arterial embolization.

The recognition of the inhibitory collaboration between heparin and antithrombin III against factor-Xa led to the introduction of low-dose heparin as prophylaxis against postoperative deep venous thrombosis. Susceptible patients are given a subcutaneous

injection of 5000 units of heparin at 8- or 12-hourly intervals. If care is taken with the injection site, local bruising is minimized, and although the systemic effects are variable, sensitive heparin assays show that detectable blood levels occur. Controlled trials have shown that such a regimen commenced before surgery and continued during the risk period of 7–10 days, very favorably influences the incidence of venous thrombosis detected with the ^{125}I fibrinogen scan method, and reduces the number of pulmonary emboli. Other procedures such as calf stimulation during immobilization for surgery, and early ambulation, are important ways of reducing venous thrombosis. Low-dose heparin appears to be superior to dextran in preventing postoperative venous thrombosis. Low-dose heparin is therefore more effective than dextran, but does not abolish thrombosis, which emphasizes the need for other physical measures, particularly in the presence of malignant disease or following major hip surgery, when heparin is comparatively ineffective.

Such prophylactic measures should be considered in particular for patients at risk, rather than for all patients. Those most likely to have thrombotic problems are the elderly, those with vascular disease, hypertension or ischemic heart disease, and malignant disease, and patients with high platelet counts or hematocrits (which should be reduced by venesection wherever possible before surgery). Pregnant women, patients taking oral contraceptives, and those with a past or family history of thrombosis should be considered to be at risk. The decision to use low or high-dose heparin depends on individual circumstances. When the indication is truly prophylatic, low-dose heparin usually suffices, but in some patients—for example, those with a past history of postoperative thrombosis—a decision may well be taken to change to full doses in the postoperative period.

Side effects of heparin are uncommon, but include bleeding, thrombocytopenia, hypersensitivity, and osteoporosis.

Treatment of Established Thrombosis with Heparin

Established thrombosis, whether venous or arterial, and embolism, prosthetic heart valves, or vascular surgery, are indications for full heparinization. Heparin is usually given by continuous intravenous infusion in doses adequate to prolong coagulation times, commonly about 30000 units over 24 hours. Precise levels necessary to achieve adequate anticoagulation are not established, but it is generally accepted that a doubling of the whole blood coagulation time over control levels, and a doubling to tripling in the activated partial thromboplastin time, reflect adequate heparin levels. The thrombin clotting time may also serve as a control test, when the therapeutic range is of the order of 25–50 seconds with a control of 10–15 seconds. However, the dosage required to achieve these levels varies between individuals and at different times in the same patient. The postoperative state, thrombocytosis, infection, and established recent thrombosis all increase the requirement for heparin. High doses of the order of 60000–70000 in 24 hours may be required for the initial period of treatment in some patients. Thereafter, the requirement usually diminishes. These variations in requirement for heparin make it important to attempt some form of laboratory control so that appropriate dosage adjustment may be made.

Duration of Treatment

Individual factors operate to determine the duration of treatment, and in many patients it may be brief and followed by a period of oral anticoagulants. Conventionally, heparin is continued for about one week before oral anticoagulation is introduced. There was no difference in efficacy or toxicity if warfarin was begun at the same time as the initial heparin treatment. In uncomplicated cases, there was more than three days' reduction in hospitalization.

Low-molecular-weight Heparin and Heparinoid Preparations

A number of preparations of low molecular weight heparin and heparinoids (including dermatan and heparan sulfate) have been developed for therapeutic use in the hope that the antithrombotic: hemorrhagic ratio might be better, and the incidence of thrombocytopenia might be lower than with conventional heparin. A single daily administration was also proposed. A number of clinical studies have been carried out, and it is likely that these agents will be in general use before long. It is still to be proven that the hoped-for advantages will be obtained.

Warfarin or Vitamin K Antagonists

When oral anticoagulants are used after initial treatment with heparin, a sufficient interval of overlap should be allowed before the optimum effect of vitamin K lack is achieved. Although the prothrombin time levels are increased within 24–48 hours of starting warfarin, this is due to an early decrease in factor-VII levels. Some days are required before the other vitamin K- dependent factors–II, XI, and X-decrease, and heparin should be continued for the 2–4 days period for these activities to decrease.

Warfarin is definitely preferred to phenindione as the oral anticoagulant of choice, because of the lower incidence of serious side effects such as skin rashes, liver damage, and bone marrow depression. Because of the interval required for effective decrease in all four coagulation factors, there is probably not much necessity for a 'loading dose' of warfarin. Warfarin at a dose of 10 mg daily is given on three successive evenings, and the thrombotest is performed on the morning of the fourth day. The predicted maintenance dosage is read from a table, the predictions being accurate if there are no confounding factors such as liver disease or cardiac failure. A similar table has been constructed for the predicted dose depending on the prothrombin ratio providing the basis for a reproducible anticoagulation regime. Subsequently, the drug is administered as a single daily dose in the range 1–25 mg, and control maintained with the prothrombin time or thrombotest.

Control of Warfarin Dose

The therapeutic range is an INR of 2.5–3.5 to be adjusted as per individual patient requirement, and for the thrombotest, an activity of 6–15%. It may be that less intense anticoagulation will be effective.

The *duration of treatment* with warfarin needs to be individually planned. In some situations, such as prosthetic heart valves, arteriovenous shunts for hemodialysis, and transient cerebral ischemic attacks, the need may be indefinite. In others, as after myocardial infarction or postoperative or stasis venous thrombosis and pulmonary embolism, the need may be short lived, and treatment for three months is adequate to allow clot dissolution and revascularization to occur. It is advisable that the need for continued oral anticoagulants be regularly reviewed in all patients. The risk of bleeding, sensitivity, and drug interactions are not inconsiderable, and should influence the decision to limit treatment to the minimal effective period.

Drug interaction between warfarin and a large number of other drugs are known to occur, and should be suspected as a cause of any inappropriate result in control tests. Also to be considered are fluctuations in diet with varying vitamin K content and individual vagaries with pill-taking. It is better to ensure that none of these alterations has occurred, and to repeat the test rather than make early adjustments in warfarin dosage.

Cessation of oral anticoagulants is associated with a temporary increase in coagulation factor levels (especially factor VII) to levels well above the normal. This may be associated

with a state of so-called 'rebound' hypercoagulability, and sometimes with rethrombosis. In the cases reported, such rebound has occurred after the anticoagulants were abruptly stopped because bleeding, and vitamin K administered. It may be preferable to reduce the dose of warfarin over 1–2 weeks when it is stopped electively, or to avoid vitamin K when warfarin is stopped abruptly because of bleeding, and treat the existing deficiency with a transfusion of fresh frozen plasma or injection of a prothrombin complex such as prothrombinex.

Thrombolytic Therapy with Streptokinase and Urokinase (Fig. 18.2)

In the search for specific treatment for thrombosis, and especially for pulmonary embolism, effective forms of induced fibrinolysis have been developed. The fibrinolytic system is based on the activation of plasminogen to the active enzyme, plasmin. Activation under ordinary conditions probably occurs largely within a formed thrombus, as the plasma contains a potent inhibitory system responsible for localizing the effects of plasmin formation. Vascular endothelium and other tissues contain activators of plasminogen, and urokinase is the activator isolated from human urine. Fibrinolytic activity can be demonstrated in normal blood, but it develops slowly and clot dissolution may take hours to days. In order to hasten this process for more convenient measurement *in vitro,* it is necessary to remove the inhibitory activity by dilution of the plasma or by extraction or fractionation procedures, as in the use of the euglobulin fraction, rich in plasminogen and its substrate, but lacking inhibitors in the euglobulin lysis time test. Extraneous activators, such as a tissue plasminogen activator, urokinase, or streptokinase, greatly enhance fibrinolysis in normal plasma and produce clot dissolution within a few minutes in adequate concentration. The effort to reproduce this phenomenon in a therapeutic sense *in vivo,* with safety, has involved a major and expensive effort in clinical and laboratory investigation.

Trials have been conducted on the use of thrombolytic therapy with streptokinase and urokinase in the treatment of myocardial infarction, venous thrombosis, arterial occlusion, and major pulmonary embolism. In the case of pulmonary embolism, the most extensive trials have been carried out and comparison made with conventional treatment with heparin. It is now established beyond reasonable doubt that both streptokinase and urokinase hasten the resolution of major pulmonary emboli, with earlier restoration of the hemodynamic abnormalities compared to heparin. This treatment may therefore be considered as an alternative or addition to heparin or surgical management, and should be considered in patients with embolism affecting more than one-third of the lung. With standard dosage regimens for streptokinase and urokinase, followed by continuous heparin in standard doses, the dissolution of pulmonary emboli is hastened. Trials conducted to date have not shown any difference in mortality in the acute phase or after 6 months of follow-up in patients treated with heparin or with either thrombolytic agent. In the majority of cases, thrombolytic therapy may not be considered to replace surgery in patients presenting with massive embolism and circulatory collapse, when

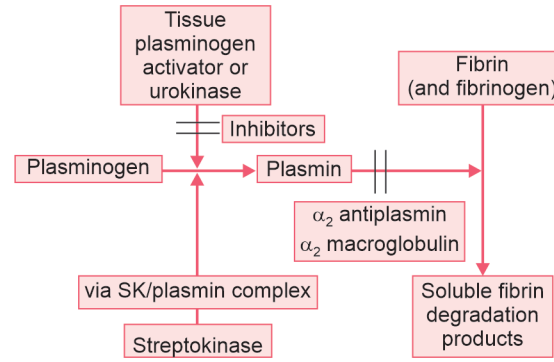


Fig. 18.2: Relationship of components of the fibrinolytic system with activators used in thrombolytic therapy

the facilities are available. If possible, the decision about the alternative forms of treatment should be made by individuals with the appropriate skills in consultation.

It is now recognized that thrombolytic therapy confers a significant advantage both in left ventricular function and mortality after acute myocardial infarction. During the 1970s, several large-scale trials of intravenous streptokinase for acute myocardial infarction were carried out. The analysis of these trials suggested a significant therapeutic effect and mortality reduction of about 20%, but the evidence was not universally accepted. Subsequently, intracoronary administration of thrombolytic agents followed by coronary angiography convincingly demonstrated 70–80% dissolution of coronary thrombus. Further knowledge gained over the period revealed that a much larger bolus dose was more effective and could be safely administered over a short period of time, and that treatment beyond 4–6 hours after onset of symptoms was likely to be ineffective. Further analysis revealed that the benefit of thrombolysis was closely related to the duration of symptoms before treatment, maximum at one hour and insignificant after 6 hours.

In deep venous thrombosis, clot dissolution is hastened and residual venous valve incompetence with dependent edema may be decreased. These long-term advantages must be weighed against the cost and dangers of thrombolytic therapy and the relative success of treatment with heparin when combined with early ambulation and vascular support. The decision about these alternatives will probably be determined by local expertise and interest.

Streptokinase is antigenic and its use often associated with mild fever. Because of the general presence of antistreptococcal antibodies, a neutralizing dose must be given before effective lytic activity can be achieved.

High doses of streptokinase produce an early state of quite severe lysis of fibrinogen in plasma and depletion of circulating plasminogen. Once so severe, and is more localized to sites of clot formation. Urokinase is not antigenic and produces lysis of clots with less systemic effect than streptokinase. However, it is very expensive, and its use is more restricted than that of streptokinase.

Recent Advances in Therapeutic Plasminogen Activators

Biochemical manipulation of streptokinase and plasminogen mixtures have resulted in therapeutic agents that show some promise. In particular, by acylating the active site serine of streptokinase–plasmin complex, a compound, BRL 26921, has been produced which binds fibrin in inactive form and, thus sited, slowly becomes activated due to de-acylation of the active site.

A more promising approach is the isolation and subsequent cloning of tissue plasminogen activator. Recombinant tissue plasminogen activator has shown considerable potential in laboratory and animal experiments, and lately in human trials. The advantage of these two products is greater specificity for fibrin, allowing fibrinolysis to take place without the development of a systemic hemorrhagic state.

Control of treatment with these agents can be readily achieved with a limited range of laboratory procedures. The thrombin clotting time is the most useful test, and prolongation reflects the presence of degradation products of fibrin in the circulation and, to a lesser extent, the reduction in the level of circulating fibrinogen. Other tests of lytic activity, such as the euglobulin lysis time, fibrin clot assay, and fibrinogen levels, may also be helpful. Bleeding complications of this treatment are not uncommon, but seldom severe. If vascular catheters are avoided and control confined to venepuncture, bleeding should not occur. Intramuscular injections must not be given. Surgery within the previous two

weeks, active peptic ulcer, or an established bleeding tendency are contraindications. Recent streptococcal infection greatly increases the resistance to streptokinase.

Thrombosis during Pregnancy

The treatment of thrombosis during pregnancy must take account of possible fetal damage, which is well documented following warfarin administration. It is advisable to avoid the use of warfarin in the first 12 weeks and in the last 2 weeks before delivery. Some researchers are opposed to warfarin at any time during pregnancy. Heparin does not cross the placenta, and can be used in high or low dose without problem. It is the preferred treatment of deep vein thrombosis and pulmonary embolism, and may be continued by low-dose subcutaneous injection if indicated for the rest of the pregnancy.

CHAPTER

19

Hypersplenism and Splenomegaly

NORMAL SPLENIC FUNCTIONS

Spleen can be considered to be a huge lymph node that has a special anatomical and physiological relation to the circulating blood.

Antibody Elaboration

Spleen, like lymph nodes shares this function. Splenectomy is known to reduce resistance to infections in man and experimental animals.

Blood Destruction and Phagocytosis

The aged and effete red blood cells are removed from circulation by phagocytic cells of the reticuloendothelial system. Spleen is an important organ for this. It also removes imperfectly formed, fragmented and damaged cells from the blood—called the culling function. It is probable, too, that spleen plays a part in removal of leukocytes and platelets from the blood at the end of their lifespan. In addition, particulate matter and organisms, bacterial, fungal, and protozoal, are removed from the circulation by phagocytosis in the spleen.

Blood Storage

In man, the amount of blood contained in the normal spleen is about 20–60 mL of red blood cells (much more in other animals). In certain disorders there can be excessive pooling of blood (harmful) in pathologically enlarged spleens. There is evience that reticulocytes are to some extent selectively concentrated in spleen. Spleen contains a pool of platelets and dynamic exchange exists between splenic and circulating platelets: the exchangeable pool in the normal spleen represents approximately 30% of the total circulating mass of platelets. The size of the splenic platelet pool is related to splenic size: an increase in size of this platelet pools is a major factor in hypersplenic thrombocytopenia. Leukocyte storage in spleen is not clearly defined. Lymphocytes constitute half the spleen cell population. Granulocytopenia is common in patients with splenomegaly.

Blood Production

In fetal life spleen provides all the blood cells, however, after birth it produces only lymphocytes and monocytes. In certain pathological conditions the spleen may undergo myeloid metaplasia and revert to its embryonic function of producing all the three cellular elements of blood.

Blood Maturation

The effect of splenectomy on blood suggests that the spleen contributes, at least partially, to the process of maturation of cells once they have been released from the bone marrow.

HYPERSPLENISM

With chronic splenomegaly in certain patients there can be pan/bi/monocytopenia—this is called hypersplenism. Contemporary evidence suggests that in the case of red cells, *Pooling* or sequestration in the spleen which is often but not invariably associated with excess destruction is the major factor in pathogenesis; expansion of plasma volume may also contribute to anemia. Similarly, an increase in the splenic platelet pool related to the increased size of the spleen is the main cause of thrombocytopenia. Another, yet not confirmed school of thought states, that spleen elaborates a splenic hormone (not yet identified) that inhibits the release of mature cells from the marrow, and sometimes arrests the maturation of developing cells.

ETIOLOGY

Causes of Hypersplenism

I. Primary (idiopathic)

II. Secondary (symptomatic)

1. Portal hypertension with congestive splenomegaly (Banti's syndrome)
2. Malignant lymphomas
3. Rheumatoid arthritis—Felty's syndrome
4. Lipoid storage disease—Gaucher's disease
5. Sarcoidosis
6. Kala-azar, chronic malarial splenomegaly, tropical splenomegaly
7. Chronic infections—tuberculosis, brucellosis, bacterial endocarditis, chronic bacteremia
8. Thalassemia
9. Chronic lymphatic leukemia, myelosclerosis.
10. Hairy cell leukemia.

DIAGNOSIS

One has to establish that hypersplenism exists and secondly one has to ascertain its cause.

Diagnostic Criteria of Hypersplenism

These are four, viz.,

1. A peripheral blood picture of anemia, neutropenia and thrombocytopenia, either singly or in combination.
2. A normally cellular or hypercellular bone marrow
3. Splenomegaly
4. Return of the peripheral blood picture to normal or near normal following splenectomy.

TREATMENT

Splenectomy produces partial or complete recovery of the abnormal blood picture in otherwise uncomplicated cases. When the effect of the hypersplenism is not sufficient to cause symptoms, splenectomy offers no benefit to the patient. Splenectomy is

indicated when significant problems are caused by the sole or the additional effect of hypersplenism in reducing the count of blood cells, usually anemia of sufficient severity to cause symptoms, neutropenia predisposing to infections, or thrombocytopenia causing spontaneous bleeding.

SPLENOMEGALY

The size of the spleen varies with the causative disorder splenomegaly is usually classified as slight (when spleen is just palpable or palpable up to 5 cm), moderate (when spleen reaches to about the umbilicus), and marked (when it extends below the umbilicus or into the left iliac fossa). Occasionally with massive splenomegaly the spleen may extend into the right iliac fossa.

CAUSES OF SPLENOMEGALY

Mild Splenomegaly

Infective Cause

- Acute, subacute and chronic infections
- Acute
  - Infectious mononucleosis
  - Typhoid fever
  - Paratyphoid fever
  - Brucellosis
  - Infectious hepatitis
  - Toxoplasmosis
  - Typhus
  - Septicemia
- Subacute and chronic
  - Bacterial endocarditis
  - Tuberculosis
  - Brucellosis
  - Syphilis
  - Histoplasmosis
  - Chronic meningococcal septicemia
- Parasitic
  - Malaria
  - Kala-azar (can cause marked splenomegaly)
  - Hydatid
  - Trypanosomiasis

Disorders in which Splenomegaly is Only Occasionally Present (Mild)

- Megaloblastic anemias
- Chronic iron deficiency anemias
- ITP
- Rheumatoid arthritis
- Hyperthyroidism
- Multiple myeloma
- SLE

- Sarcoidosis in early stages
- Amyloidosis
- Disorders mentioned below cause moderate or marked enlargement in the early stage or following treatment
- Idiopathic, no demonstrable cause.

Moderate Enlargement

- Malignant lymphomas
- Chronic lymphocytic leukemia
- Chronic granulocytic leukemia
- Acute leukemia (early stage)
- Portal hypertension with congestive splenomegaly
- Chronic hemolytic anemias
- Polycythemia vera
- Essential thrombocythemia
- Myelodysplastic disorders.

Marked Enlargement

Usual or Common

- Hairy cell leukemia
- Myelosclerosis
- Chronic myeloid leukemia
- Tropical splenomegaly, kala-azar, bilharziasis, chronic malaria
- Thalassemia major (children)
- Splenic cysts and tumors
- Gaucher's disease

Less Common

Disorders listed under moderate enlargement, especially lymphomas and congestive splenomegaly.

INDICATIONS FOR SPLENECTOMY

Disorders in which Splenectomy is Usually Indicated

- Hereditary spherocytosis
- Chronic ITP
- Symptomatic hypersplenism
- Portal hypertension due to splenic vein thrombosis
- Hairy cell leukemia.

Disorders in which Splenectomy is Sometimes Indicated

- The malignant lymphomas
- Thalassemia major
- Autoimmune acquired hemolytic anemia
- Acute ITP
- Felty syndrome
- Hereditary elliptocytosis
- Tropical splenomegaly

Disorders in which Splenectomy is Occasionally Indicated

- Myelosclerosis
- Chronic lymphocytic leukemia
- Thalassemia major
- Aplastic anemia.

EFFECTS OF SPLENECTOMY

Hematological Effects

Changes given below refer to those that occur in normal subjects after splenectomy. Consequences of splenectomy for pathological conditions on one or more of the cellular elements may be substantially modified by the effect of the underlying disorder on the production of blood cells by the bone marrow. In autoantibody-mediated hemolysis or thrombocytopenia, for example, the relative increase in the respective cell counts following splenectomy is often significantly increased.

Red Cells

Target cells usually appear in the blood film, and the cells have increased resistance to hemolysis in the osmotic fragility test. Occasional spherocytes may be detected. Small remnants of nuclear material, Howell-Jolly bodies, characteristically are present in red cells, as they are no longer selectively 'pitted' from circulating red cells by the spleen. Other inclusions, such as aggregates of iron-containing material, may also be observed. Changes of this type often persist indefinitely after splenectomy.

White Cells

After splenectomy, there is an increase in the total white cell count within several hours, and it usually reaches a maximum after a day or two. The count then gradually falls over a period of weeks or months in the uncomplicated situation, to normal values. In some cases, a mild increase, e.g. up to 15×10^9/L, persists for many years. The maximum reached by the white cell count is usually about twice normal, although it may be higher. The sharp rise in the count is due mainly to an increase in neutrophils, but after a few weeks to months, the neutrophil count falls to near normal levels, and the count of circulating lymphocytes and monocytes rises, and remains increased, apparently permanently. There may also be a slight increase in eosinophils and basophils. In splenectomized subjects, leucocytosis in response to infection is characterized by a greater than normal shift to the left, with the appearance of myelocytes.

Platelets

The platelet count rises sharply, often within a matter of hours, reaches a peak in 1–2 weeks, and then usually falls to normal values over a period of weeks or months. In about one-third of cases, it remains raised indefinitely. This is especially so in patients with continuing hemolysis, and this group has a reported increased risk of thromboembolism. The maximum platelet count is usually 3–4 times normal, and on occasion reaches $2000–3000 \times 10^9$/L.

The current view is that changes after splenectomy are a reflection of the capacity of the spleen to sequester cells newly released from the bone marrow. The spleen contributes to the maintenance of normal red cell appearance by its 'pitting' function—the selective removal of particles from the cytoplasm of the red cell without injuring the cell.

SPLENIC ATROPHY

Changes similar to those occurring after splenectomy have been described accompanying congenital absence or acquired atrophy of the spleen. Disorders in which acquired atrophy may occur include sickle cell disease, coeliac disease, dermatitis herpetiformis, and essential thrombocythemia.

CLINICAL EFFECTS

The spleen is not essential for life. In otherwise healthy adults it can be removed without apparent alteration of health or longevity.

Susceptibility to Infection after Splenectomy

Splenectomy does not appear to render normal adults more susceptible to infection. However, there is evidence in infants and young children of an increased incidence of severe, and sometimes fatal, infections after splenectomy. It appears that children under the age of three years are most frequently affected, particularly infants under 1 year. The interval between splenectomy and the onset of infection has been less than 3 years in most reported cases, but may be longer. The *Pneumococcus* has been the organism most commonly isolated (>50% of cases) but infections with group A *Streptococcus, Hemophilus influenzae,* enteric bacteria, and other organisms have also been reported. No increase in incidence of viral infections has been noted. Reported infections include septicemia, meningitis, pneumonia, pericarditis, and acute endocarditis. A feature of many infections has been their fulminating character. With septicemia and meningitis, death may occur within 12–24 hours of the onset of symptoms. It has been shown that some cases of severe infection are associated with acute disseminated intravascular coagulation. Although the incidence of infection in children over the age of 3 years does not appear to be much increased, when it does occur, infection may be severe and overwhelming. It is recommended that the young splenectomized patient has close supervision for several years postoperatively, so that immediate several years postoperatively, so that immediate and energetic treatment can be instituted in the event of sudden and severe infectious illness.

Investigators have pointed out that the nature of the disorder that led to the splenectomy influences the risk of postoperative infection. Infection is not markedly increased following splenectomy for trauma, hereditary spherocytosis, idiopathic thrombocytopenic purpura, Gaucher's disease, and portal vein thrombosis with congestive splenomegaly, while it is more common in other disorders such as thalassemia major, lymphoma, leukemia, hepatitis with portal hypertension, and diseases requiring treatment with corticosteriods in relatively high doses. Septicemia of an unusually fulminant nature is sometimes encountered in hyposplenism due to sickle cell disease. Prophylactic immunization with pneumococcal vaccine should be considered in individuals at increased risk. However, even when polyvalent vaccines are employed, septicemia may still occur. Long-term prophylaxis with penicillin may therefore be needed in susceptible patients.

CHAPTER

20

Bone Marrow Transplantation

This is usually carried out between siblings (allogeneic transplantation) who are HLA-identical and whose cells do not react in a mixed lymphocyte culture or can be shown to be matching by a number of different techniques of DNA analysis. Syngeneic (twin), haploidentical and HLA-matched but unrelated donors may also be used in appropriate cases.

INDICATIONS

BONE MARROW (OR PERIPHERAL BLOOD STEM CELL) TRANSPLANTATION

Allogeneic (or Syngeneic) (Fig. 20.1)

- Severe aplastic anemia
- Acute myeloid leukemia
- Acute lymphoblastic leukemia
    i. Poor prognosis: first remission
    ii. Second remission or subsequently
- Chronic myeloid leukemia
- Other severe acquired disorder of the marrow, e.g.
    - Myeloma
    - Myelodysplasia
    - Paroxysmal nocturnal hemoglobinuria
- Benign inherited disorders, e.g.
    - Thalassemia major
    - Sickle cell anemias
    - Severe combined autoimmune deficiency
    - Mucopolysaccharidosis
    - Severe Gaucher disease (type II)

Autologous (Fig. 20.2)

- Malignant lymphoma (Hodgkin's or non-Hodgkin's) usually post first relapse.
- Other solid tumors, e.g. breast cancer
- Acute myeloid or lymphoblastic leukemia in first or subsequent remission with or without marrow purging
- Myeloma
- For gene therapy, e.g. adenosine deaminase deficiency.

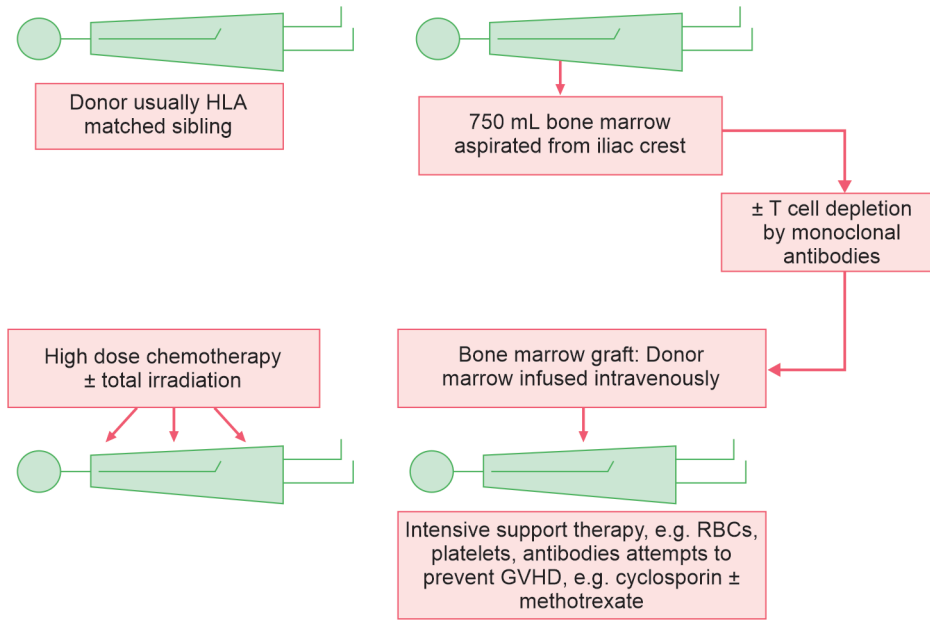

Donor usually HLA matched sibling
750 mL bone marrow aspirated from iliac crest
± T cell depletion by monoclonal antibodies
High dose chemotherapy ± total irradiation
Bone marrow graft: Donor marrow infused intravenously
Intensive support therapy, e.g. RBCs, platelets, antibodies attempts to prevent GVHD, e.g. cyclosporin ± methotrexate

Fig. 20.1: Allogeneic bone marrow transplantation

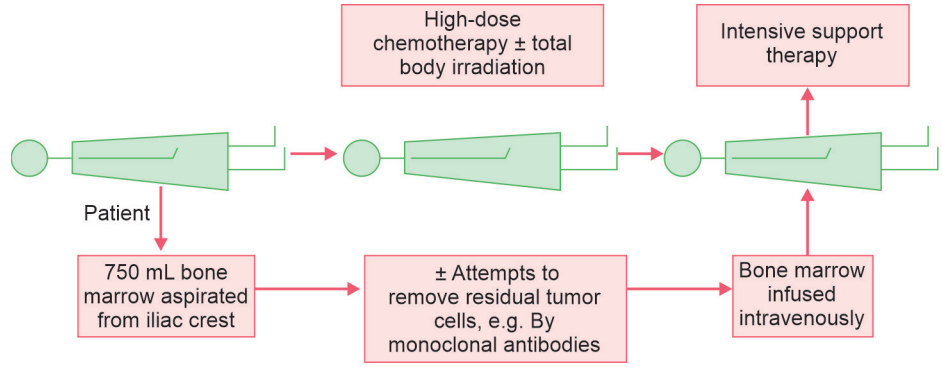

High-dose chemotherapy ± total body irradiation
Intensive support therapy
Patient
750 mL bone marrow aspirated from iliac crest
± Attempts to remove residual tumor cells, e.g. By monoclonal antibodies
Bone marrow infused intravenously

Fig. 20.2: Autologous bone marrow transplantation

The recipient has severe aplastic anemia, poor-prognosis leukemia (for example, myeloid leukemia in first remission, acute lymphoblastic leukemia in second or subsequent remission, or in first remission with poor prognostic features, or chronic myeloid leukemia in chronic phase), a severe genetic abnormality (for example, thalassemia major) or a severe acquired marrow disorder (for example, myelodysplasia. myeoma or paroxysmal nocturnal hemoglobinuria).

In autologous bone marrow transplantation, the patient's own bone marrow is used to rescue the patient from profound marrow ablation caused by high-dose chemotherapy, with or without total body irradiation, for malignant disease.

Peripheral stem cells may be harvested using a stem cell separator and used instead of bone marrow for an allogeneic and autogenous transpiration. Recovery of platelets appears to be enhanced by the technique.

Bone marrow transplantation has also been carried out for certain benign congenital or acquired abnormalities of the marrow (for example, thalassemia major a paroxysmal

nocturnal hemoglobinuria), or no lymphoid system (as in severe combined immune deficiency) or of the macrophage system (as in Hulen's disease).

Failure of engraftment is unusual in well-matched transplants for leukemia but is more common when the recipient has aplastic anemia or when donor and recipient are not fully matched.

COMPLICATION OF BONE MARROW TRANSPLANTATION

EARLY (<100 DAYS)

Acute GVHD

- Skin
- Liver
- Intestines
- Infection
  - Bacterial
  - Fungal
  - Herpes simplex
- Cytomegalovirus

Graft failure
Hemorrhagic cystitis
Interstitial pneumonitis
Veno-occlussive disease

LATE (>100 DAYS)

Chronic GVHD

- Scleroderma
- Sicca syndrome
- Arthritis
- Hepatitis
- Malabsorption
- Pulmonary lesion

Chronic pulmonary disease
Autoimmune disorders
Cataracts
Infertility
Leukoencephalopathy
Secondary malignancy

Infections are a major hazard during the post-transplant period. Attempts at presentation include reverse-barrier or laminar flow nursing, prophylactic non-absorbable antibiotics and antifungal agents, and early use of systemic antibiotics for febrile episodes. Prolonged antibiotic therapy, however, increases the likelihood of fungal infections. Hemopoietic growth factors may be used to enhance granulocyte recovery.

Cytomegalovirus infection may result from reactivation of a previous latent infection or growth transmission of the virus in blood products, and may result in severe pneumonitis.

Herpes simplex virus infection is a frequent complication. which tends to become generalized and may cause pneumonia, encephalitis or skin lesions. Infection can be prevented by the use of prophylactic acyclovir. Pneumonia due to *Pneumocystis carinii* is another frequent complication of the immunosuppression and neutropenia.

Total body irradiation itself may cause side effects involving epithelial strictures; there is damage to nails and nailbeds and temporary complete alopecia.

Veno-occlusive disease (VOD) of the liver is an acute condition due to chemotherapy and radiotherapy. Preceding chemotherapy any abnormal liver function may also predispose to veno-occlusive disease.

GRAFT VERSUS HOST DISEASE (GVHD)

This is another major post-transplant development because of the reaction of the immunocompetent cells in the graft against the tissues of the host, causing graft versus host disease (GVHD) which may be acute (occurring in the first hundred days post-transplant) or chronic. There is triad of skin, mucous membrane and gut, and liver involvement which is classified according to severity into grades I, II, III and IV. In acute cases of GVHD there is a widespread erythematous itchy skin rash which tends to be particularly severe on the hands and feet. In severe cases there may be a bullous eruption and subsequent widespread exfoliation.

In chronic cases the lesions tend to be firm, red and plaque-like and ultimately may, in some patients, form a scleroderma-like picture with contractions and ulceration.

The mucous membrane may also be affected, with formation of lichen planus-like lesions in the mouth and pharynx. The histological appearances of GVHD are normally seen in life after skin or rectal biopsy. In acute GVHD the skin shows inflammatory changes with death of epidermal cells and a lymphoid infiltrate leading in severe cases, to denudation.

The rectal mucosa also shows death of epithelial (crypts) cells and inflammatory changes when severe, there is loss of small and large intestinal mucosa.

Liver function is abnormal in acute and chronic GVHD, except in mild cases. The histological appearances includes damage to bile duct epithelial cells, inflammatory changes and cholestasis. GVHD can now largely be prevented in HLA-matched allogeneic transplantation if T lymphocytes are completely removed in vitro from donor marrow.

A frequent post-transplantation complication is an interstitial pneumonia, which is more common in GVHD may also be related to lung irradiation and to infection, particularly with cytomegalovirus.

CHAPTER

21

Immunodeficiency Disorders

The main types are primary and secondary immunodeficiency diseases (Table 21.1). In severe combined immunodeficiency disease (SCID), the T- and B-lymphocyte systems fail to develop. There is severe lymphopenia and hypogammaglobulinemia. Affected infants fail to thrive and die early in life from recurrent infections, such as by *Pneumocystis carinii* cytomegalovirus, other viruses, fungi, and bacteria. There is atrophy of the thymus: the lymph nodes and spleen are small and devoid of lymphoid cells. The most common cause is deficiency of enzyme adenosine deaminase (ADA). Deficiency of another enzyme, purine nucleoside phosphorylase, causes a more selective lack of T cells. ADA deficiency has been treated successfully by bone marrow transplantation and, most recently by 'gene therapy'—in which the ADA gene has been introduced *in vitro* into the patient's lymphocytes which have been reinfused.

In the very rare syndrome of lymphoreticular dysgenesis, there is failure of development of both the reticuloendothelial and lymphoid systems. Affected infants die soon after birth from overwhelming infection. There is marked lymphopenia, and stigmata of splenic

Table 21.1: Immunodeficiency disorders

Primary	*Secondary*
B cell • X-linked hypogammaglobulinemia (Bruton type immunodeficiency) • Purine nucleoside phosphorylase (PNP deficiency) • Selective IgA or IgM deficiency • Common variable immunodeficiency disease	*B cell* • Myeloma • Nephrotic syndrome • Protein-losing enteropathy
T cell • Thymic hypoplasia	*T cell* • HIV infection • Hodgkin's disease • Non-Hodgkin's lymphoma • Drugs, e.g. steroids, azathioprine
Mixed B and T cell • Severe combined immunodeficiency disease (SCID) due to ADA deficiency • Bloom's syndrome • Wiskott–Aldrich syndrome • Lymphoreticular dysgenesis	*Mixed B and T cell* • Chronic lymphocytic leukemia • Chemotherapy • Radiotherapy] with or without marrow transplantation

atrophy may be found in the peripheral blood which shows large numbers of Howell–Jolly bodies in RBCs.

ACQUIRED IMMUNE DEFICIENCY SYNDROME (AIDS)

This syndrome is caused by infection with the human immunodeficiency virus (HIV), a retrovirus of the lentivirus subgroup.

HIV produces its predominant effects through infection of T-helper ($CD4^+$) cells. Some $CD4^+$ cells are lyzed directly by replicating HIV, but the virus remains latent in most host cells, unrecognized by the patient's immune system. When such latently infected T cells are activated, virus replicates and cell necrosis follows. The CD4 antigen appears to be the main receptor for HIV, and CD4 antigen-presenting cells are also an important site for viral replication. Direct infection by the virus of other cells, for example brain cells, also accounts for some of the pathology of the disease.

Transmission of the virus is usually by sexual contact, or by blood or blood products. AIDS is particularly common in homosexual men but is now widely seen in heterosexuals too. It is also seen in intravenous drug abusers, hemophiliacs and other patients requiring multiple blood transfusions, or people receiving other human blood derived products (made from HIV infected blood).

The clinical outcome of infection has been classified into four stages or groups. The factors which determine the progression from one stage to another remain uncertain. A prodromal period of about 6 weeks follows initial infection symptoms resembling infectious mononucleosis which may then occur. A proportion of patients pass through the asymptomatic and persistent lymphadenopathy stages to the AIDS related complex (ARC) and fully developed AIDS. The sequence of expression of HIV antigens and different antibodies following primary infection is given in Fig. 21.1.

Examination of involved lymph nodes reveals characteristic abnormalities. There is progressive depletion of CD4 cells, and the peripheral blood shows a lymphopenia and an alteration in the T-lymphocyte subsets, with a fall in the $CD4^+$ (helper suppressor) ratio from the normal value of 1.5–2.5:1 to < 1:1. A polyclonal rise in serum immunoglobulins is often found in some cases with a paraprotein present. The diagnosis is confirmed by detection of antibodies to one or other surface antigen, or by detection of the antigens themselves. Hematological abnormalities may include anaemia, neutropenia or thrombocytopenia; these are often autoimmune in origin but are sometimes due to direct infection of hemopoietic stem and progenitor cells in the bone marrow. The cytopenias may be due to dysplastic changes, marrow lymphoma, or fibrosis. A wide spectrum of opportunistic organisms cause infections in AIDS patients, including *Pneumocystis carinii*, cytomegalovirus, atypical mycobacteria, *Cryptococcus*, histoplasmosis, and leishmaniasis, often nonspecific granuloma are found. A proportion of patients develop Kaposi's sarcoma, a vascular skin tumor while

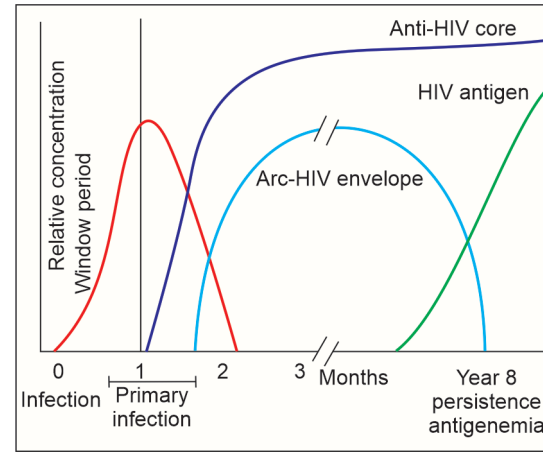


Fig. 21.1: Sequence of expression of HIV antigens and different antibodies following primary infection

others may develops non-Hodgkin's, lymphoma, which is likely to be high grade and with a 20% incidence of lymphoma in the central nervous system.

CLINICAL STAGES OF INFECTIONS WITH THE HIV VIRUS

Group 4 is subdivided into the AIDs-related complex (ARC: generalized lymphadenopathy with persistent fever; weight loss; unexplained diarrhea; hematological abnormalities; CNS manifestations and full blown AIDS with severe opportunistic infections and/or neoplasms. An absolute CD4 count of $<0.2 \times 10^9$/L in the peripheral blood implies AIDS has developed).

Group 1

Acute Infection

Infectious mononucleosis type illness: fever, lymphadenopathy, arthralgia, myalgia, mild meningoencephalitis, and myelopathy. Diagnosis confirmed by seroconversion.

Group 2

Asymptomatic infection with positive serology

Anemia: Neutropenia, thrombocytopenia: CD4 counts, hypergammaglobulinemia.

Group 3

Persistent generalized lymphadenopathy.

Group 4

Acquired immune deficiency syndrome (AIDS)

i. *Constitutional symptoms:* Fever, malaise, weight loss, diarrhea.
ii. *Neurological symptoms:* Peripheral neuropathy, myelopathy, AIDS dementia complex.
iii. *Secondary infections*
   a. Pulmonary: *Pneumocystis*, cytomegalovirus: *Cryptococcus*: Atypical mycobacteria, e.g. *Mycobacterium avium*.
   b. Gastrointestinal: *Candida*, mycobacteria, *Salmonella*, cryptosporiadiae, herpes simplex virus, cytomegalovirus.
   c. Skin: *Candida*, herpes simplex virus, herpes zoster.
   d. CNS: *Cryptococcus*, CMV, *Toxoplasma*.
iv. *Malignant tumors:* Kaposi's sarcoma, non-Hodgkin's lymphoma, squamous carcinoma of oral and rectal mucosa.
v. CD4 cells $<0.2 \times 10^9$/L

HEMATOLOGICAL MANIFESTATIONS OF HIV DISEASE

Fall in CD4 lymphocyte counts and lymphopenia. Anemia, neutropenia, thrombocytopenia—single or combined. Bone marrow changes.

- Hypercellular (increased plasma cells and lymphocytes)
- Normocellular
- Hypocellular
- Dysplastic changes of normoblasts, granulocytes, megakaryocytes
- Increased fibrosis, plasma cells
- Granulomas (AFB, *Cryptococcus*, uncertain origin)
- Other infections, e.g. *Histoplasma*, leishmanii, *Penumocystis* (may need culture to demonstrate)

- Benign nodules
- Infiltration by lymphoma

  Low serum B_{12} and folate levels

  Toxic changes due to drugs, e.g. megaloblastosis due to azidothymidine, pentamidine

  Plasma protein changes
  - Polyclonal rise in IgG
  - Paraprotein.

OVERVIEW OF HIV/AIDS

HUMAN IMMUNODEFICIENCY VIRUS

Since its recognition in 1981, the HIV pandemic has spread unabated across continents. The catastrophic potential of the pandemic may still not have been fully realized as it continues to exact enormous toll throughout the world in both human and economic terms.

The demographic characteristics of those affected by the epidemic has changed drastically since the first cases were reported in the year 1981 in the USA comprising overwhelmingly of gay population. Today new cases of HIV infection originate predominantly through heterosexual contacts and injecting drug users. The infection rates amongst women, adolescent girls and children have increased dramatically. It is estimated that a total of 40 million people worldwide are living with HIV/AIDS and at least 5 million people were infected with HIV and at least 3 million people perished due to the disease. Summary for the HIV/AIDS epidemic is shown in Fig. 21.2.

The epidemic has negated decades worth of work on controlling diseases, improving nutrition and in many African countries it has become an unprecedented emergency with approximately 25% of the most productive age group between 15 years to 49 years being infected.

It is estimated that in sub-Saharan Africa the life expectancy would reduce from average 64 years to 47 years. If the present warning of epidemiologists go unheeded, South Asia, South East Asia and China may follow the disastrous course of sub-Saharan Africa. The

People infected with HIV	Total Adults Women Children <15 years Total	5 million 4.3 million 1.8 million 800,000 40 million
No. of people living with HIV/AIDS	Adults Women Children <15 years Total	37.2 million 17.6 million 2.7 million 3 million
AIDS deaths	Adults Women Children <15 years Total	2.4 million 1.1 million 580,000 21.8 million
Total no. of AIDS deaths since the beginning of the epidemic	Adults Women Children <15 years	17.5 million 9 million 4.3 million
Total no. of AIDS orphans since the beginning of the epidemic	Total	13.2 million

Fig. 21.2: Summary of the HIV/AIDS epidemic

battle against AIDS has to be fought on multiple fronts such as education, counseling, prevention of infection, voluntary testing, treatment and vaccination programs.

HIV Virus

The HIV-1 and HIV-2 belong to the retrovirus family, subfamily lentivirinae. Both retroviruses are so named because one step of their replication cycle is the creation of DNA copy of their RNA genome. The genetic information of a retrovirus therefore flows from RNA to DNA at this step, which is in the opposite direction of the flow in most biological systems. HIV is a lipid enveloped positive stranded RNA virus like all retroviruses.

RNA viruses are distinguished by the possession of an enzyme viral reverse transcriptase that transcribes the viral RNA into provirus DNA which is integrated into the host cell genome.

Origin of HIV

Lentiviruses other than HIV have been found in a wide range of nonhuman primates. As a result of molecular analysis and analogy, it is now generally thought that the HIV-2 is similar to SIVsm (Sooty mangabey monkey) and the HIV-1 is similar to the SIVcpz (Pantroglodytes troglodytes chimpanzees), indigenous to the West and West Central Africa.

It is postulated that through zoonosis these viruses at some point of time crossed over to the humans.

HIV Virion

The mature HIV-1 virion measures approximately 100 nm in diameter. The HIV-1 genome consists of two identical, noncomplementary sense (+) strands of RNA, each approximately 9.2 kb long, packaged in a cone-shaped protein core, composed of 24 kDa capsid (CA) proteins (p24), surrounded by a unique bilayer envelope derived from the host cell membrane. The viral enzymes, reverse transcriptase, integrase and protease are packaged with the RNA in the core. The surface (SU) and transmembrane (TM) glycoproteins, gp 120 and gp 41 respectively, form a noncovalent complex. Also incorporated into the envelope are various host proteins including HLA class I and II DR molecules.

Regional HIV/AIDS Statistics

Region	*Epidemic started*	*Adults and children living infected with HIV/AIDS (million)*	*Adults and children newly infected with HIV (million)*	*Percent Adults prevalence rate*	*Percent of HIV positive adults who are women*	*Main mode(s) of transmission for adults living with HIV/AIDS*
Sub Saharan Africa	Late 70s Early 80s	28.1	3.4	8.4	55	HTS
North Africa and the Middle-East	Late 80s	0.44	0.080	0.2	40	HTS, IDU
South and South East Asia	Late 80s	6.1	0.8	0.6	35	HTS, IDU
East Asia and Pacific	Late 80s	1.0	0.27	0.1	20	IDU, HTS, HMS

Contd.

Regional HIV/AIDS Statistics						
Region	*Epidemic started*	*Adults and children living infected with HIV/ AIDS (million)*	*Adults and children newly infected with HIV (million)*	*Percent Adults prevalence rate*	*Percent of HIV positive adults who are women*	*Main mode(s) of transmission for adults living with HIV/AIDS*
Latin America	Late 70s Early 80s	1.4	0.13	0.5	30	IDU, HMS, HTS
Caribbean	Late 70s Early 80s	0.42	0.06	2.2	50	HTS, HMS
Eastern Europe and Central Asia	Early 90s	1.0	0.25	0.5	20	IDU
Western Europe	Late 70s Early 80s	0.56	0.03	0.3	25	HMS, IDU
North America	Late 70s Early 80s	0.94	0.045	0.6	20	IDU, HMS, HTS
Australia and New Zealand	Late 70s Early 80s	15,000	500	0.1	10	HMS
Total		40	5	1.2	48	

HTS = Heterosexual, HMS = Homosexual, IDU = Injecting Drug Users

Biologically HIV-2 is very similar to the HIV-1, with the genome exhibiting about 60% homology in the gag and pol genes and about 30–40% homology in the other viral genes. It is recognized that HIV-2 is less easily transmitted than HIV-1 and the period between infection and illness is relatively longer in the case of HIV-2. While the HIV-1 is widespread globally, the HIV-2 is primarily found in West Africa.

HIV Types and Variants

The HIV virus is a term used to refer collectively to the HIV-1 and HIV-2 retroviruses which are distinct from one another.

The HIV-1 genome (Figs 21.3 and 21.4) is heterogeneous and the envelope gene with its 5 hypervariable region V3 of the surface glycoprotein gp 120, encoding a central loop structure contains the most important site for antibody binding. The phylogenetic analysis of V3 sequences have lead to the identification of three major groups of

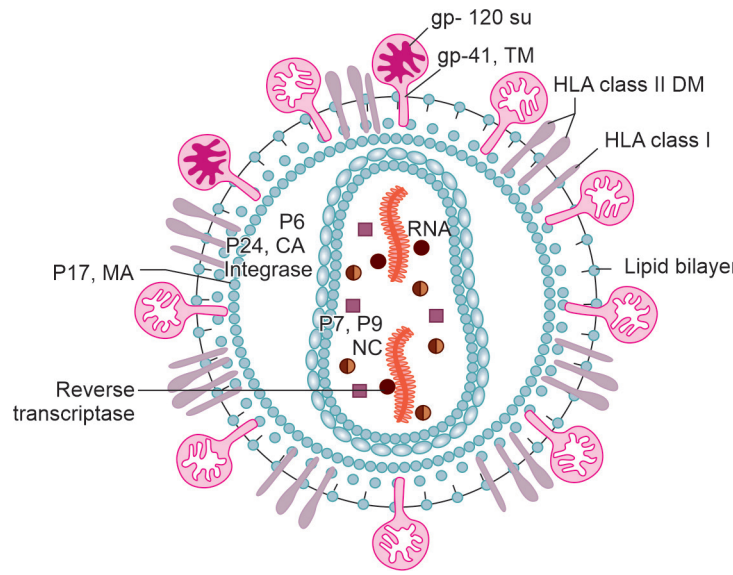


Fig. 21.3: HIV genome

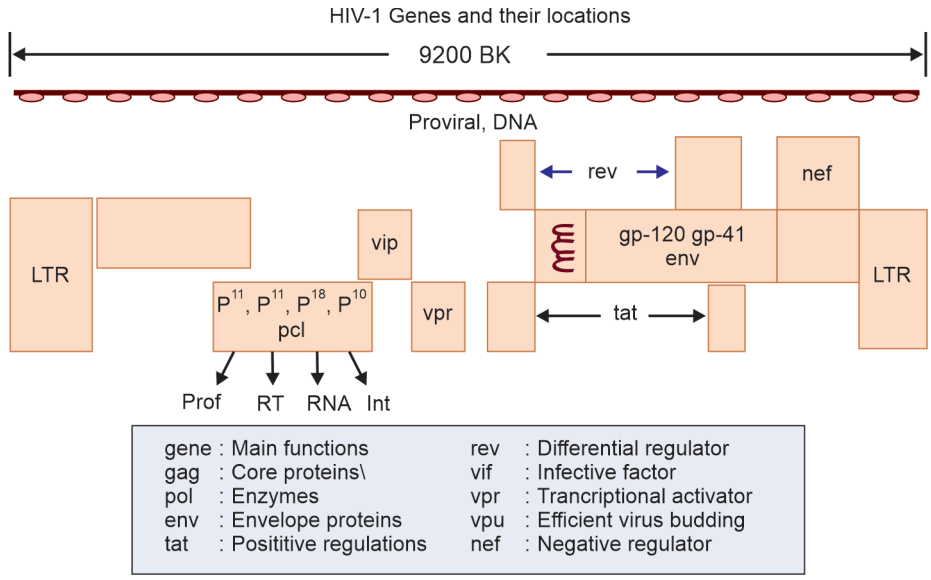


Fig. 21.4: Genetic map of HIV

HIV-1: M (major), O (outlier) and the most recently described group N (New). Functions of various HIV genes is shown in Fig. 21.7.

Within the major group M, there are nine subtypes A through to K. As the virus continues to mutate and the global epidemic continues, new subtypes are expected to develop and be isolated.

The viruses originally identified as subtype E and I are now considered intersubtype recombinants.

The group O, of HIV-1 shares 55–70% homology with the major group M of HIV-1.

In Africa, most subtypes are found, although, subtype B is relatively less prevalent. The geographic distribution of HIV-1 subtypes is shown in Fig. 21.5.

Life Cycle of the HIV

Like all lipid enveloped viruses, the HIV must remain moist to be infectious and it is easily inactivated. For this reason, HIV is primarily transmitted sexually, by exchange of

Sr.	*HIV-1 Subtypes*	*Geographical distribution*
1	B	USA, Japan, Australia
2	A, D	Sub-Sahara Africa
3	C	Africa, India
4	E	Central African Republic
5	F	Thailand, South East Asia, Brazil, Romania
6	G, H	Russia, Central, Africa
7	I	Cyprus
8	O	Cameroon

Fig. 21.5: Geographic distribution of HIV-1 subtypes

blood, or from mother to child perinatally or by breastfeeding or through sharing infected needles during injecting drug use.

After initial contact and attachment to the hosts immune system (e.g. lymphocytes, monocytes) post-infection a cascade of intracellular events within the host are unraveled, the end product being the production of new viral particles, death of the infected cells and the ultimate devastation of the hosts immune system. The main steps in the HIV replication cycle (Fig. 21.6) are as follows:

Attachment to the Lymphocyte Membrane

On the surface membrane of all living cells are complex protein structures called "receptors". A receptor is often compared to a lock into which a specific key or "ligand" will fit. There are at least two receptors on T-lymphocytes to which the human immunodeficiency virus (HIV) locks on to. The primary receptor is called "CD4". But a second receptor that loops through the cell membrane seven times is critical for infection to occur.

HIV infection of a lymphocyte requires attachment of the virus to the cell membrane through both of these "ligand receptor" links. In cells whose "7-transmembrane receptor" is different, the HIV "key" no longer matches the lymphocyte "lock" and attachment is incomplete. Such cells may avoid infection with HIV.

Entry of the Viral RNA

Tight attachment of the viral particle to receptors on the lymphocyte membrane enables fusion with the cell membrane. The viral contents including viral RNA then empty into the cell's cytoplasm, like the other viruses.

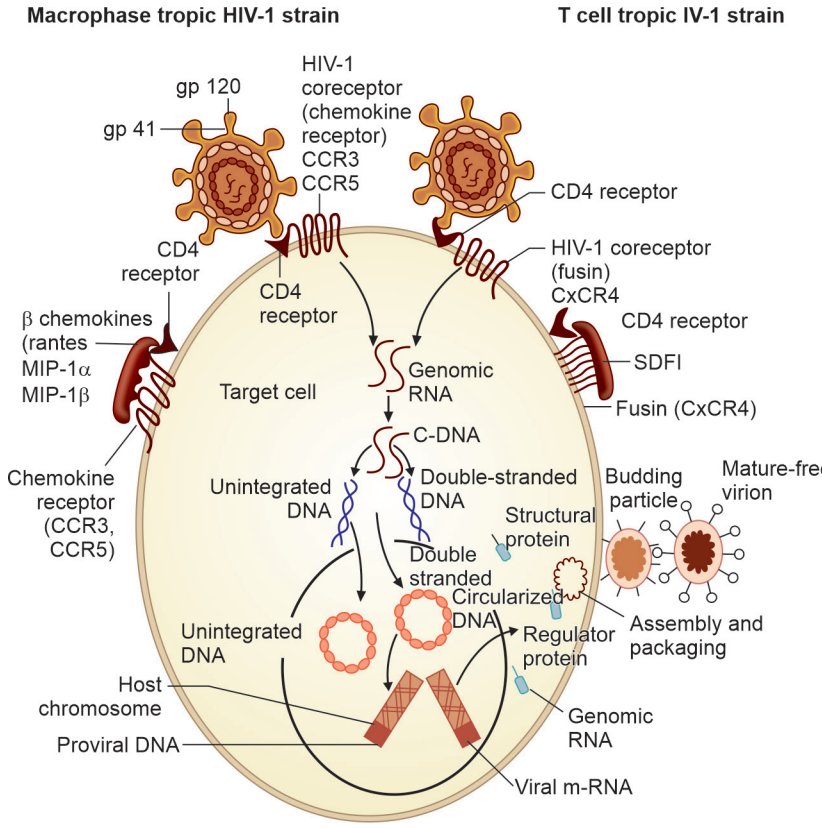


Fig. 21.6: The HIV replication cycle

HIV gene	*Protein product*	*Function in life cycle of HIV*
gag	Gag/core protein precursor	Viral core proteins: p24: major capsid protein p17: matrix protein p9: binds to viral RNA p7: binds to viral RNA
env	Env./envelope proteins	Viral envelope proteins: gp 120: major envelope protein, mediates virion binding to cell surface receptor (CD4) gp 41: mediates fusion of viral envelope and cell membrane
pol	Reverse transcriptase	Converts single-stranded viral RNA into viral DNA duplex
	Integrase	Integrates viral DNA duplex into host cell genome as provirous DNA
	Protease	Cleaves core precursor polyprotein into functional core proteins
tat	Tat protein	Essential regulatory protein, transactivates expression of all viral genes
rev	Rev protein	Regulatory protein, activates expression of HIV structural and enzymatic genes
vif	Vif protein	Role in viral budding and infectivity of free virions
vpu	Vpu protein	Promotes release of budding virions from host cell
nef	Nef protein	Regulatory protein (essential for pathogenicity of SIV)
vpr	Vpr protein	Regulatory protein, role uncertain

Fig. 21.7: Functions of the HIV gene

Reverse Transcription

Converting viral RNA into DNA.

An enzyme that is part of the human immunodeficiency virus reads the sequence of viral RNA nucleic acids that have entered the host cell and transcribed the sequence into a complementary DNA sequence. This enzyme is called "reverse transcriptase". Without reverse transcriptase, the viral genome cannot be incorporated into the host cell, and cannot reproduce.

Reverse transcriptase sometimes makes mistakes reading the RNA sequence. The result is that not all viruses produced in a single infected cell are alike. Instead, they end up with a variety of subtle molecular differences in their surface coat and enzymes. Thus through each replication cycle HIV surface molecules are continually changing.

Integration of Viral DNA

Once the viral RNA has been reverse-transcribed into a strand of DNA, the DNA can then be integrated (inserted) into the DNA of the lymphocyte. The virus has its own enzyme called "integrase" that facilitates incorporation of the viral DNA into the host cells DNA. The integrated DNA is called a provirus.

Transcription: Back to RNA

As long as the lymphocyte is not activated or "turned-on", nothing happens to the viral DNA. But if the lymphocyte is activated, transcription of the viral DNA begins, resulting in the production of multiple copies of viral RNA. This RNA codes for the production

of the viral proteins and enzymes (translation) and will also be packaged later as new viruses.

Translation: RNA → Proteins

There are nine genes in the HIV RNA. Those genes have the code necessary to produce structural proteins such as the viral envelope, core and the enzymes like reverse transcriptase, integrase, and another crucial enzyme called protease.

Viral Protease

When viral RNA is translated into a polypeptide sequence, that sequence is assembled in a long chain that includes several individual proteins (reverse transcriptase, protease, integrase). Before these enzymes become functional, they must be cutoff from the longer polypeptide chain. Viral protease cuts the long chain into its individual enzymes components, which then facilitate the production of new viruses.

Assembly and Budding

Finally, viral RNA and associated proteins are packaged and released from the lymphocyte surface, taking with them a swatch of lymphocyte membrane containing viral surface proteins. These proteins will then bind to the receptors on other immune cells facilitating continued infection and propagation.

Budding viruses are often exactly like the original particle that initially infected the host, however, the resulting viruses also exhibit a range of variations.

During its replication cycle, the HIV is capable of mutating at a rate of approximately one nucleotide substitution per genome per replication cycle; making the virus capable of wide genetic diversity and flexibility to respond to a wide array of selection pressures.

As a consequence, drug resistance and immunologic escape mutants are rapidly generated in infected persons through all stages of infection.

The natural history of HIV infection is shown in Fig. 21.8. The duration of clinical latency following primary HIV infection varies widely among individual persons depending upon many factors; such as exposure categories (blood, sexual contact, mother-to-child), portal of entry, viral pathogenicity and mutation, host resistance, intrinsic and specific immune responsiveness, new treatment strategies with combinations of potent antiretroviral drug therapy, etc.

There are also some HIV-seropositive, long-term "nonprogressors" who have continued to be healthy without evidence of disease or immune defects for ten or more years after primary HIV infection.

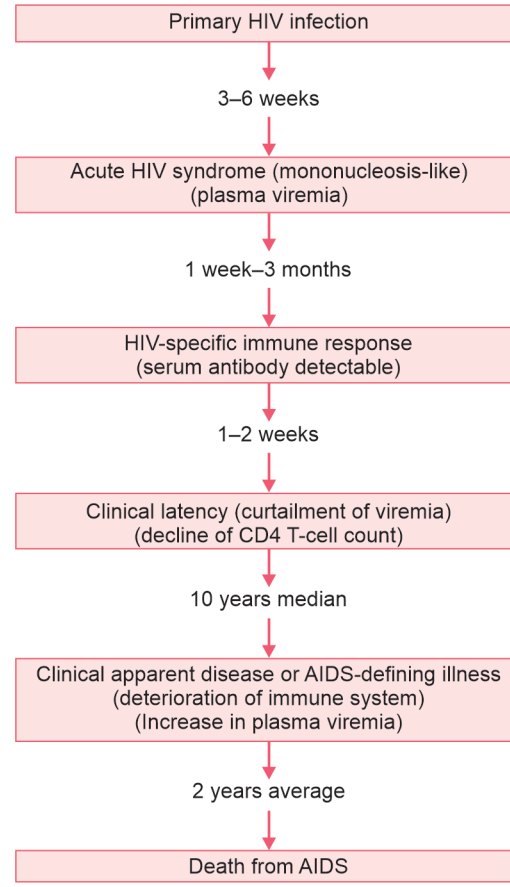


Fig. 21.8: Natural history of HIV infection

Although reliable clinical data are available for only the past 15 years or so, it is predicted that approximately 95% of HIV-1-infected patients will progressively develop AIDS within 15 years of infection. Immunopathogenesis of HIV infection is shown in Fig. 21.9. The first few weeks after primary infection are characterized by an acute mononucleosis or flu-like illness. During this stage seroconversion usually occurs and is associated with a rapid increase in circulating viral titers. This is followed by virus dissemination to lymphoid organs. Adaptive host immunity, including neutralizing antibodies and cytotoxic T lymphocytes (CTL) against virus-infected cell, subsequently develop. While these responses may temporarily control the infection, generally they are unable to eliminate the virus. The patient then enters the stage of clinical latency or the asymptomatic period. A progressive decrease in CD4+ peripheral blood lymphocytes (PBL) continues during the latency stage. When CD4 counts fall below 200/mm^3, the disease usually enters a symptomatic phase, characterized by opportunistic infections and other AIDS-defining conditions in the host.

Clinically Apparent Disease

This clinically apparent, symptomatic stage is a consequence (secondary manifestation) of the progressive and profound deterioration of the immune system that occurs over time in most patients with HIV infection. The CD4 T-cell count continues downward to the range of 200–400/mm^3. Plasma viremia and p24 antigenemia approach high levels such as seen in the primary infection. Some of the constitutional symptoms, opportunistic infections, and other manifestations of advanced symptomatic HIV disease are given in the Fig. 21.10.

As the CD4 T-cell counts continue to fall, sometimes precipitiously, and reach levels that define AIDS (CD4 T-cell count less than 200/mm^3), predisposition to AIDS-defining conditions, such as opportunistic infections caused by viruses, bacteria, fungi and protozoa,

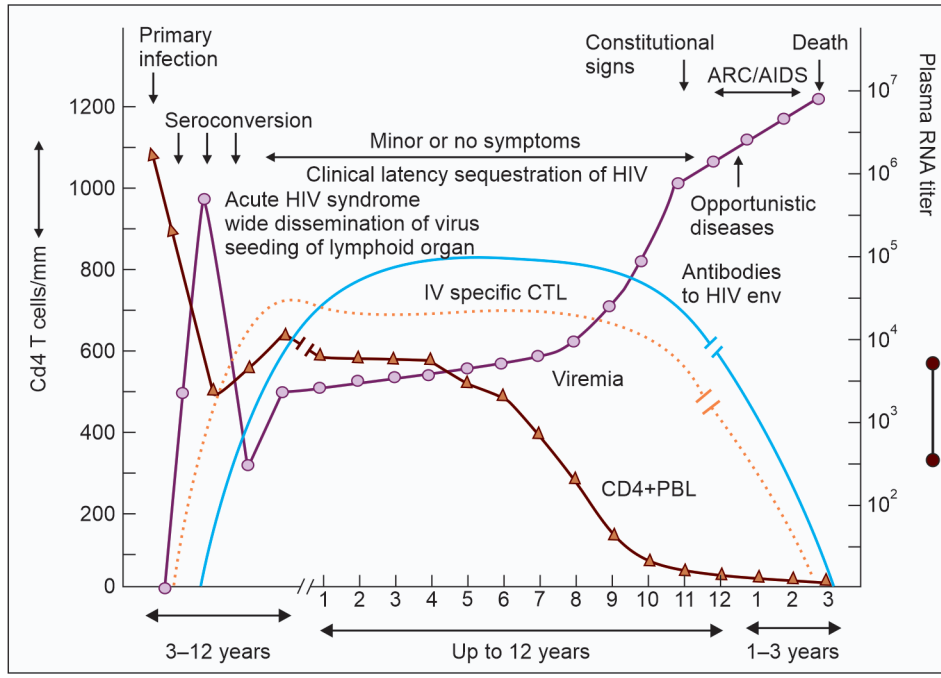


Fig. 21.9: Immunopathogenesis of HIV infection

(CD4T—cell 200-400/mm³)
1. Constitutional symptoms: ▪ Fever ▪ Weight loss ▪ Fatigue ▪ Night sweats ▪ Diarrhea ▪ Persistent generalized lymphadenopathy
2. Infections: ▪ Oral or vaginal candidiasis ▪ Oral hairy leukoplakia ▪ Herpes zoster (shingles) ▪ Herpes simplex ▪ Listeriosis
3. Other manifestations: ▪ Cervical dysplasia ▪ Cervical carcinoma *in situ* ▪ Idiopathic thrombocytopenic purpura (ITP) ▪ Neuropathy ▪ Seborrhea

Fig. 21.10: Manifestations of advanced symptomatic disease

neoplastic disease, HIV encephalopathy, wasting syndrome, and progressive multifocal leukoencephalopathy that complicate the clinical course and are often the cause of death.

HIV Specific Prognostic Markers

Several HIV specific markers can be used for disease prognosis. Of these declining levels of antibody to p24 (anti-p24) and increasing concentrations of HIV antigen, especially the p24 antigen have been most widely used.

Levels of antibody/antigen, however, can vary widely between individuals and therefore sequential tests are usually necessary to confirm a trend. Indeed some patients are not measurably antigenemic until later on in the disease progression, whereas others may be antigenemic for many years. There is still a debate as to the mechanism of these changes. A decline in anti-p24 levels could lead to reduced formation of antigen–antibody complexes and hence allow detection of the p24 antigen. Alternatively, the developing p24 antigenemia may reflect increased virus replication. As the infection progresses, the amount of viral RNA and infectious virus in plasma increases and the proportion of peripheral blood mononuclear cells carrying the provirus also increases.

The prognostic significance of p24 antigenemia is indicated by a four-fold increase to the progression to AIDS within 3 years, in those who show circulating p24 antigen. However, only about 50% of patients with ARC and approximately two-thirds of patients with AIDS will be antigenemic.

A decrease in titer of anti-p24 antibody is also associated with disease progression and has been shown to occur before the rise in p24 antigen levels. The decline in titer of anti-p24 antibody may therefore be an early indicator of disease progression. List of conditions in the 1993 AIDS Surveillance Case Definition by Center for Disease Control and Prevention are shown in Fig. 21.11.

List of conditions in the 1993 AIDS surveiliance case definition issued by center for disease control and prevention

1. CD4 T-cell count <200/mm^3
2. Opportunistic infections:
   - Candidiasis of bronchi, trachea, or lungs
   - Candidiasis, esophageal
   - Coccidioidomycosis, disseminated or extrapulmonary
   - Cryptococcosis, extrapulmonary
   - Cryptosporidiosis disease (other than liver, spleen, or nodes)
   - Cytomegalovirous retinitis (with loss of vision)
   - Herpes simplex: chronic ulcer (s) (>1 month duration); or bronchitis, or esophagitis
   - Histoplasmosis, disseminated or extrapulmonary
   - Isopsoriasis, chronic intestinal (>1 month duration)
   - *Mycobacterium avium* complex or *M. kansasii*, disseminated or extrapulmonary
   - *Pneumocystis carinii* pneumonia
   - Pneumonia, recurrent
   - *Salmonella*, septicemia, recurrent
   - Toxoplasmosis of brain
3. Neoplastic disease:
   - Cervical carcinoma, invasive
   - Kaposi's sarcoma
   - Lymphoma, Burkitt's (or equivalent term)
   - Lymphoma, primary in brain
4. HIV encephalopathy (AIDS dementia complex)
5. Wasting syndrome due to HIV
6. Progressive multifocal leukoencephalopathy (PML)

Fig. 21.11: List of CDC conditions

Nonspecific Markers of HIV Progression (Fig. 21.12)

Markers other than HIV specific antigens and antibodies can be of use in predicting and assessing progression to AIDS.

At present the most commonly used marker is the CD4 cell count, expressed either as an absolute value (normal adult range 600–1700/mm^3) or as a CD4:CD8 ratio (normal adult ratio 1.2–3.5).

Similarly, when CD4 cell counts fall to 200/mm^3 or less, prophylaxis against *Pneumocystis carinii* is generally initiated.

Measurements of cell counts are, however, subject to potentially large biological and laboratory variation and interpretation should be based on more than one result. A recent

- CD4 cell count
- CD4/CD8 ratio
  - CD8 cell count
  - Serum interferon
  - Serum IgA
  - Serum IgE
- Serum β_2-microglobulin
  - Serum/urine neoptrin

Fig. 21.12: Nonspecific markers of HIV progression

prospective study has suggested that combining measurements of the levels of either serum β_2-microglobulin or neopterin along with the CD4 cell count improves its prognostic significance. It is likely that a combination of markers rather than a single marker will prove useful for the monitoring of disease progression.

The precise role and practicality of this approach required for the evaluation.

Serological profile of HIV infection is shown in Fig. 21.13. Following infection there is evidence of active viral replication as virus can be isolated from cultures of peripheral blood mononuclear cells and p24 antigen can be detected in the serum by immunoassays and viral RNA based assays. This antigenemia may only last for a week or two and disappears when the antibody response develops following seroconversion. The timing of collection of any blood samples is crucial as prior to or at seroconversion antigen is detected in less than 50% of samples. Seroconversion occurs in most patients by 4–8 weeks after exposure although in some cases antibody may not appear until 6–9 months later. This delay between infection and appearance of antibodies is termed as the "window period". At seroconversion, antibody of the IgM class can be detected. Analysis of the reactivity of the IgG response by immunoblotting or enzyme immunoassay based on recombinant proteins shows that antibodies develop rapidly to the principal structural proteins, e.g. the envelope glycoproteins gp 160, gp 120 and gp 41 and the core proteins p18, p22. Antibody to the polygene proteins can also be detected. Antibodies to the envelope glycoproteins gp 160, gp 120 and gp 41 incase of HIV-1 and gp 36 in case of HIV-2 infections may appear before antibodies to the core proteins although the time difference is generally only a matter of days or a few weeks.

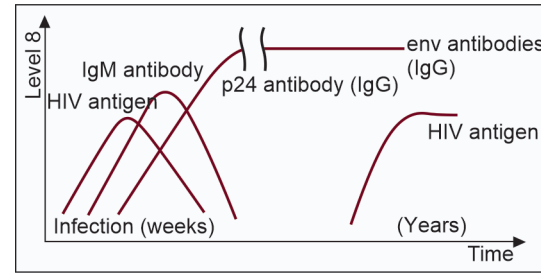


Figure represents the idealized sequence of serological events during HIV infection progressing AIDS

Fig. 21.13: Serological profile of HIV infection

After seroconversion there is evidence of continued virus replication as virus can be cultured from the plasma as well as detected by PCR based RNA assays. Indirect evidence is provided by the progressive increase in the level of antibodies to all the major virus components during this time.

In neonates the serological profile is complicated by the presence of the maternal anti-HIV antibody.

For some years after seroconversion the patient may remain well or develop persistent generalized lymphadenopathy. Throughout this period viremia may increase. A reduction in the level of antibody to the p24 core protein and the appearance of p24 Ag are associated with progression to clinically overt disease. In the later stages of AIDS, as the immune system is destroyed, antibody may not be detectable in some patients.

HIV Diagnostic Testing Techniques

As has been discussed that virtually all individuals who become infected with HIV mount an IgG antibody response to the virus which current immunoassays will detect by 4–8 weeks after primary infection and in the majority of cases for the remainder of the individuals life. Screening and diagnostic tests thus rely upon the detection of antibodies to HIV. During part of the window period IgM anti-HIV and HIV Ag may be present, but specific screening for them though desirable is not cost effective in low prevalence populations.

During the search for the causative agent of AIDS, tests for anti-HIV detection were developed. Initial testing was by Western blot analysis. However, the magnitude of the HIV epidemic and the need for extensive screening were not fully understood. By 1983 and 1984, it had become apparent that rapid, simple and inexpensive assays would be required. Research assays were developed and in 1985 several manufacturers introduced enzyme immunoassays for blood donor screening. In the ensuing years these assays have evolved rapidly by utilizing products of modern biotechnology, for screening and confirmation of HIV infection.

Enzyme Immunoassays (EIA)

The diversity of EIA formats make a simple, meaningful definition difficult to formulate, but EIAs can be described as a binding assays that depend upon the antigen–antibody reaction as the basis and the enzyme reaction as a marker for the proof of reaction. Anti-HIV EIA kits in various formats are available. Obtaining high quality results not only depends upon the quality of the kit but also on strict adherence to kit protocols, well-maintained equipments and good performance assurance procedures. Under these conditions many assays currently available will achieve >99% sensitivity and specificity.

Typically, a batch of tests takes 1.5–3 hours to complete. Since the EIAs detect anti-HIV antibodies, the solid phase on the microwell is coated with HIV specific proteins.

Generation of Assays

The terms first, second and third generation have been applied to anti-HIV assays according to the source of the HIV Ag used on the solid phase as opposed to sequential improvements.

First generation assays may lack specificity because of reactions with contaminating cell proteins and lack sensitivity due to the high sample dilution needed to attain acceptable specificity. For second and third generation assays, the identification and sequencing of important antigenic sites, the expression of proteins of precisely targeted portions of HIV cDNA, and the synthesis of oligopeptides containing immunodominant epitopes has provided new antigens for use in the assay systems. When incorporated into EIAs these recombinant proteins and peptides have led to substantial improvements in sensitivity and specificity and have the added advantage of being safer, easier and more reproducible in performance.

Generation of Anti-HIV Assays	
First generation culture	Native viral antigens from lymphocyte
Second generation	Expression products of recombinant HIV cDNA
Third generation	Chemically synthesized oligopeptides (approximately 15–40 amino acids) Double antigen sandwich assays

EIA Assay Formats

There are four basic assay formats for the detection of antibodies to HIV.

Type 1: Indirect or Antiglobulin (Fig. 21.14)

A relatively crude native HIV antigen preparation is immunoabsorbed to the solid phase by pre-coating with HIV antibody. Antihuman globulin conjugated to enzymes is used as a tracer, with a compatible substrate as a chromogenic marker.

Type 2: Competitive Assay (Fig. 21.15)

In competitive assays the sample need not be diluted. These assays exhibit high sensitivity. To ensure good specificity and sensitivity the spectrum of anti-HIV detected is predominantly against env. determinants, but because antibodies to env antigens of HIV-1 and HIV-2 show only limited cross reaction the competitive format is unsuitable for combined anti-HIV-1/HIV-2 screening.

Type 3: Class Specific Antibody Capture Assay (Fig. 21.17)

Class specific antibody capture assays are only available as a research tool. They have proved to be highly accurate for detecting anti-HIV specimen such as saliva and urine and find important applications in epidemiological research and other situations where venipuncture is impractical.

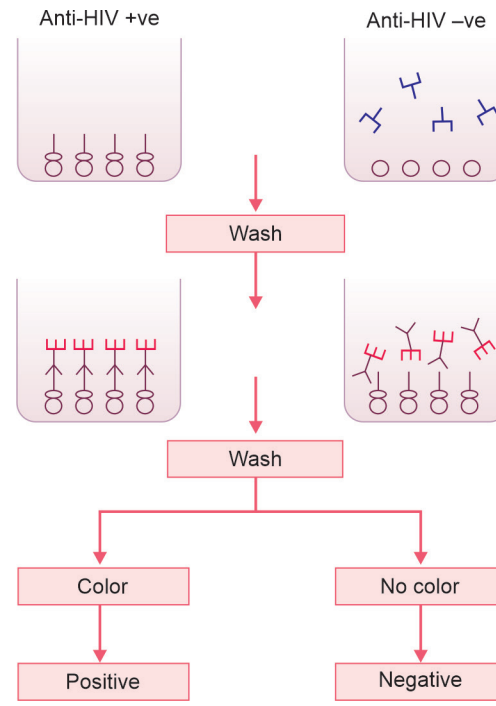


Fig. 21.14: Indirect or antiglobulin assays

Type 4: Double Antigen Sandwich or Immunometric Assay (Fig. 21.16)

A commercially available example of this assay detects anti-HIV-1 and anti-HIV-2 by using recombinant HIV-1 antigens and synthetic oligopeptide antigens from the HIV-2.

These assays are sensitive and specific, an added advantage being that they do react with IgM anti HIV antibodies and they are reported to detect seroconversion earlier.

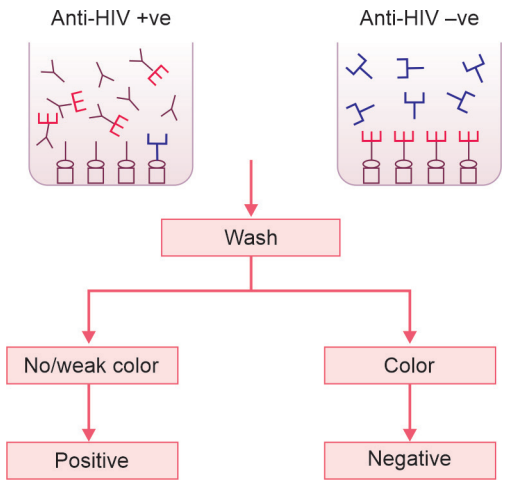


Fig. 21.15: Competitive assays

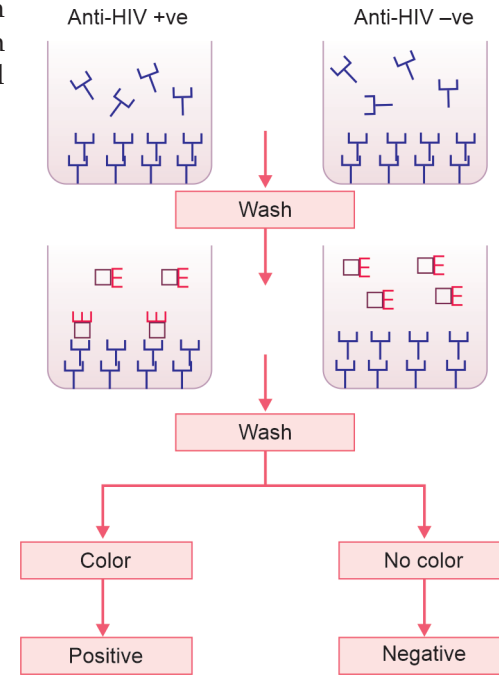


Fig. 21.16: Immunometric assays

HIV Antigen EIAs

HIV Antigen EIAs have been developed primarily for the purpose of monitoring HIV-infected individuals for antigenemia, but are also used to monitor growth of HIV in lymphocyte cultures. The methods used are in general modifications of antibody sandwich assays described earlier.

Anti-HIV prepared from the serum of HIV infected individuals, animal antisera raised against purified viral or recombinant antigens and monoclonal antibodies have all been used in various combinations as capture and detector antibody as well as a variety of enzymes and substrates.

The sensitivity of HIV Ag assays can be below 50 pg/mL. It should be noted, however, that standards do vary from one commercial kit to another reflecting the unsuitability of standardizing by weight of a complexed antigen, which will be, measured immunometrically.

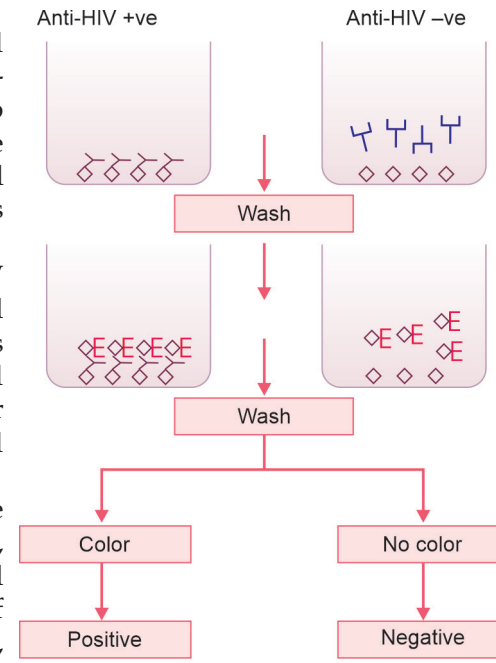


Fig. 21.17: Class specific antibody capture assays

Estimations of HIV antigen level can, therefore, be regarded as 'semiquantitative'. Most manufacturers do provide HIV antigen standards, which can be included in assay runs in order to produce a standard curve from which specimen HIV antigen levels can be extrapolated. Despite the 'semiquantitative' nature of the estimation, sequential monitoring of titers may be informative regarding disease progression or efficacy of antiviral therapy.

Confirmation of positive findings is recommended and some manufacturers have reagents available for this purpose, usually a blocking antibody.

Confirmatory/Supplementary Assays

Confirmatory tests are used to confirm whether specimens found reactive with a particular screening test contain antibodies specific to HIV. The most commonly used confirmatory test until recently, was the Western blot (WB). However, its use has proven to be very expensive and prone to producing large numbers of indeterminate results. The differences in criteria for the interpretation of Western blot also has lead to classification difficulties of indeterminate samples. Now, studies have shown that combinations of ELISAs or simple/rapid assays can provide results as reliable as the WB at a much lower cost.

1. Western Blot

The Western blot was developed to detect antibodies to HIV and to qualitatively determine to which viral proteins the antibody response had been raised. The assay is based on the detection of the reaction of specific antibodies in the sample with viral antigens that have been electrophoretically separated and transferred ("blotted") onto a nitrocellulose sheet on a backing. Antibodies, which react with antigens on the membrane, are visualized with an enzyme labeled antihuman immunoglobulin and an enzyme substrate, which gives an insoluble coloured product at the site of reaction.

Although WB allows the humoral immune response to HIV to be mapped, there are several problems: Not all sera from infected individuals react with all HIV proteins. Sera from some uninfected individuals may react with one or more HIV proteins or with contaminating residual lymphocyte cell proteins. Large differences between batches and particularly between manufacturers occur in the number of proteins and their relative concentrations and positions on the strips. The assay is read visually. To counter these difficulties, some standardization has been attempted, particularly in terms of interpretation of the reactivity observed. Several sets of criteria have been recommended which differ in details, but commonly require an antibody to at least one env. gene product, and at least one band from gag and/or pol for classifying a positive test result.

Results which neither satisfy 'positive' nor 'negative' criteria are termed as 'indeterminates'.

Recently, immunoblots, which resemble WB but utilize a selection of synthetic HIV antigens applied to membrane strips as discrete bands have been developed (LIA/RIBA). These are reportedly comparable to the electrophoterically manufactured traditional blots, with reduction in the proportion of indeterminate results. However, these are equally expensive.

2. Indirect Immunofluorescence

It has been commonly employed to detect anti-HIV antibodies. Acetone fixed HIV infected lymphocytes are incubated with sample, followed by a fluorescein conjugated antihuman IgG. The cells are then examined using fluorescent microscopy. If anti-HIV is present a typical pattern of fluorescent staining is seen in the cells. It is usually reserved for confirmation as it is time-consuming and requires highly-skilled operators and an expensive, well-maintained fluorescence microscope.

3. Radioimmunoprecipitation Assay

RIPA is used by very few laboratories. Initially, HIV Ag is metabolically labeled by substituting one or more S tagged amino acids in HIV culture medium. The labeled cells are then disrupted to release the labeled proteins and the resulting suspension is clarified. The labeled proteins are used for testing the sample. Containment facilities for handling live HIV and radioisotopes are required along with expensive equipment and consumables for immunochemistry, limiting its usage to few reference laboratories.

Virus Detection Methods

Virus isolation has traditionally been achieved by cocultivation of gradient purified patient's peripheral blood lymphocytes with stimulated lymphocytes from an uninfected individual. To seek evidence of viral replication the cultured cells may be examined by microscopy for cytopathic changes and by HIV immunofluorescence. The culture medium may also be assayed for HIV p24 Ag or reverse transcriptase activity. Isolation of HIV indicates beyond any doubt that the subject is infected with HIV. However, it has a very restricted role to play in confirmatory testing because of its relatively low sensitivity and the cost and complexity of the technique. HIV can sometimes be isolated from fresh or properly stored plasma, but usually a fresh blood sample will be required to provide peripheral blood mononuclear cells for cocultivation. Very few laboratories claim nearly 100% HIV isolation from all anti-HIV positive subject, but most achieve 60–80% success. Virus isolation is generally reserved for research purposes due to its expense, low sensitivity, long incubation time and need for high containment facilities.

p24 Antigen and Nucleic Acid Technologies (PCR)

A number of other assays have been introduced in recent years which assist in the establishment of the diagnosis of HIV infection and may also be used to monitor the progress of the infection and the response to therapy. These include assays that detect virus particles, e.g. the HIV p24 antigen ELISA, or the presence of HIV viral nucleic acid sequences (RNA or DNA) by means of nucleic acid amplification techniques. NAT assays have made it possible to detect minute amounts of viral material—as little as a single viral genome (the detection limit for most assays is around 300 copies/mL). These sensitive procedures are well suited for early diagnosis of mother-to-child transmission and for monitoring the viral load of patients who are on antiretroviral therapy. However, in routine practice this technique can have limited specificity, needs sophisticated equipment, rigorous laboratory conditions and highly trained staff, and is still very expensive.

Rapid Tests for the Detection of Anti-HIV Antibodies

Advances in technology have now made it possible to design rapid HIV antibody assays based on agglutination, immunodot, immunochromatographic and immunoconcentration/filtration techniques, the assays have comparable sensitivity to equivalent generation EIAs.

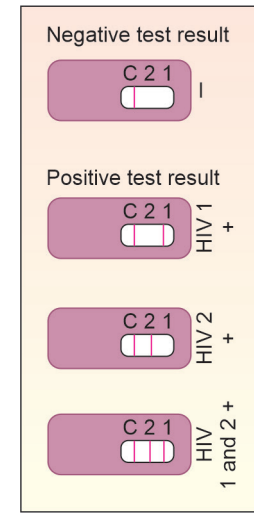
The assays are easy to perform and ideal for low through put laboratories. Since same day results are offered through the use of these tests, they help in timely detection and treatment intervention of HIV infection.

The overall considerations in rapid test assay development are as follows:

1. The tests should pick up antibody response to HIV-1 and HIV-2.
2. The test when used in areas endemic with variants of HIV 1 such as O and M (Cameroon, Gabon) should be reactive with the antibodies to the variants.
3. The test should increase the sensitivity of detection during the "window period" shortening it as much as possible.
4. The antigenic configuration of the test should preferably use recombinant antigens or peptides from the conserved immunodominant regions of the HIV-1 and HIV-2 genome.
5. The screening test should have sensitivity and specificity in excess of 99%.

Well designed rapid tests based on protein A based tracers that pick up IgG class anti-HIV antibodies bound to the solid phase, in general achieve such specifications. However, since protein A based assays necessarily map only IgG class anti-HIV antibodies, they tend to be less sensitive than systems having the ability to pick up IgG and IgM class of Anti-HIV antibodies. Third generation rapid tests now utilize the principle of double antigen sandwich immunoassays. In the double antigen sandwich system recombinant proteins from the immunodominant regions of the HIV genome are used on the capture as well as on the tracer reagent. Antibodies to HIV are thus specifically captured and sandwiched during the test specifically between the solid phase and tracer antigens. This third generation system not only improves specificity but also improves sensitivity of the assay since not only IgG but also IgM antibodies react in the system, improving the test sensitivity in the early seroconversion phase of the HIV infection. Such improved third generation rapid tests are being made available now.

Flow-through Technology



Appearance of only one control band corresponding to control region 'C'

In addition to the control band 'C', appearance of reactive band at test region '1': Specimen positive for antibodies to HIV 1.

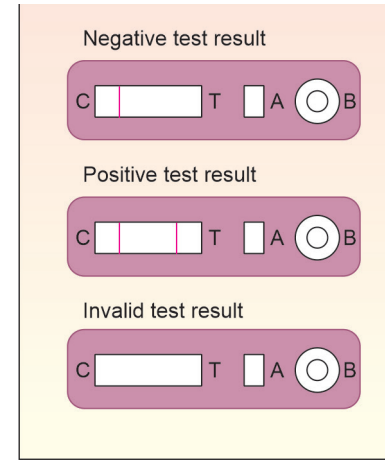
In addition to the control band 'C', appearance of reactive band at test region '2': Specimen positive for antibodies to HIV 2.

In addition to the control band 'C' appearance of reactive band at test region '1' and test region '2': Specimen positive for antibodies to HIV 1 and HIV 2.

Invalid test result

The test should be considered invalid if neither the test band nor the control band appears.

Lateral Flow Technology



Appearance of only one colored band at the control region 'C'.

Positive test result

Appearance of a coloured band at the test region 'T' in addition to the band at control region 'C'.

Invalid test result

The test should be considered invalid if neither the test band nor the control band appears.

Flow-through v/s Lateral Flow Technology

Rapid tests currently available are based either on the second generation protein A based flow through systems or the third generation lateral flow based double antigen system. The flow through technology based tests are multicomponent, multistep based systems that require cold storage. These tests also require higher degree of laboratarian involvement and can only use serum or plasma as a sample. Since the membrane needs to be rehydrated before use there is a limit on the number of tests that can be run at a time simultaneously. New generation flow through assays, however, have been reported with distinct advantages over second generation flow through assays.

On the other hand lateral flow technology based double antigen sandwich assays are single step/two step and easier to use. Suitability of whole blood as a sample and room temperature storage in addition to higher clinical sensitivity due to anti-HIV IgM detection capability make these tests a method of choice for most settings. These assays have an overall advantage overflow through technology based tests.

Application of HIV Testing

The donor screening tests for anti-HIV have been also adopted for clinical diagnosis and for epidemiological prevalence and monitoring studies. Because the consequences of a positive diagnosis of HIV infection are so serious it is generally considered that people

who are tested should be asked to give consent to testing only after counseling, which in simple and sensitive terms explains the background to the screening test, the implications of a positive finding and advice on risk avoidance. Follow-up clinical and psychological support for those found to be infected is also essential. Of equal importance is a mechanism for distinguishing which of those samples that react in screening assays are genuine, or true positives.

Screening tests for anti-HIV-1 will not detect all HIV-2 infections and the discovery of HIV-2 infections outside of its recognized endemic regions in West Africa has influenced the decision by many countries to introduce tests which can detect antibody to HIV-2 reliably. There are now many 'combined' HIV-1/HIV-2 EIAs and rapid tests that have become the screening method of choice. Simultaneously test that can in addition detect the antibody to subgroup 'O' are considered "complete" as a screening test.

Screening

Anti-HIV screening assays were primarily developed for blood bank screening with the objective of protecting the recipients of blood or its products from infection with HIV. In addition to screening, vetting of donors and phasing out of inducements to donate are now strongly recommended by WHO. However, in regions where HIV has spread primarily by heterosexual contacts, vetting of donors is difficult as "high-risk" groups are not easily recognized and more reliance is therefore placed on anti-HIV screening. Nevertheless, anti-HIV screening alone is insufficient to ensure that the blood supply is free from HIV contamination. Even if the assays were 100% sensitive, technical and clerical errors can lead to reporting errors. Many considerations affect the choice of screening assay including sensitivity, specificity, ease of performance and cost. Poor sensitivity may jeopardize the safety of recipients of blood or other tissues while, on the other hand, poor specificity is costly in terms of unnecessarily wasted donations, the cost of extraconfirmatory testing and the problems of management of donors whose samples are reactive. Limited resources have led some transfusion services to consider the screening test simply as a means to protect recipients. Many however, are also able to assume responsibility for their donors, ensuring that screen-reactive samples are confirmed before counseling the donor and offering referral to an expert HIV clinic.

Worldwide application of anti-HIV screening assays outside the strictly clinical arena are increasing: Many life insurance companies require anti-HIV screening before offering cover to applicants, and some authorities require HIV testing before issuing marriage licences, immigration documents or admitting recruits to armed services. Some controversy surrounds these applications of HIV screening with respect to their moral, ethical and legal justification. If carried out, confirmation of a screen reactive finding is an absolute requirement, especially as these tests are applied to populations in which HIV prevalence is generally low and therefore false positive reactions are common relative to genuine positive reactions.

Diagnosis

When screening tests for anti-HIV first made it possible to identify HIV-infected individuals who did not manifest the classical signs and symptoms of AIDS, there was some debate whether testing was worthwhile for them. However, improvements in predicting the progression of disease by use of prognostic markers and continuous improvements in treatment, both with antiretroviral and prophylactic treatment of opportunistic infections, now favor the policy of early identification of all HIV-infected individuals.

There are three main diagnostic applications of anti HIV testing:

a. To confirm that a patient presenting with symptoms associated with HIV infection is infected with HIV
b. To investigate a person who has been exposed to infection because of exposure to body fluids from a known HIV-infected individual, perhaps by sexual intercourse or by inoculation.
c. And to test those who belong to one of the recognized risk groups, are healthy and have no definite history of exposure, but are anxious and present themselves for testing in the hope of gaining the reassurance of a negative result.

Many diagnostic laboratories now take the precaution of running two screening tests (usually different manufacturers kits) on each sample to decrease the possibility of false-negative findings, whether due to a technical or clerical error or some inherent fault in an assay.

Approach to Confirmatory Supplementary Tests

The best of the currently available anti-HIV screening assays will give rise to less than 1 in 1,000 repeatable false-positive reactions. Although this represents excellent specificity, when such a test is applied to a low prevalence population only a small proportion of reactive samples will be genuine. To avoid the potentially serious consequences of a false diagnosis, several strategies have been devised for further testing to determine the authenticity of a positive screening reaction. This is referred to as confirmatory or supplementary testing. It is generally accepted that only positive and unexpected negative results in screening assays need to be confirmed. So that unnecessary confirmatory testing is not undertaken, most laboratories first retest reactive samples twice or more in the screening assay. Unrepeatable reactions are reported as negative. Whatever confirmatory strategy is employed, it is good practice to seek a follow-up sample from any individuals found to be reactive. This is to primarily safeguard against a false diagnosis due to technical or clerical errors, but also to check that an uncomfirmable positive reaction on a screening assay is not a result of sampling too early during seroconversion. Cases of dual infectivity with HIV-1 and HIV-2 have been reported and thus in some instances this may require confirmation (Fig. 21.18).

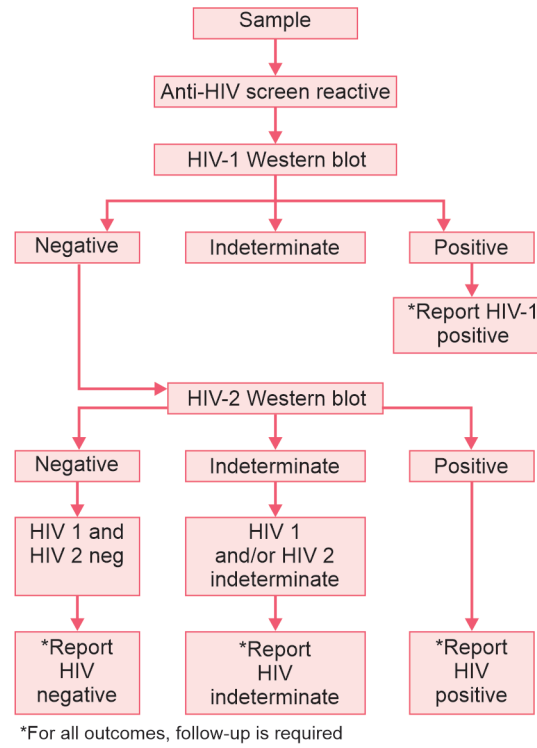


Fig. 21.18: Approach to confirmatory tests

Western Blot (WB)

The most widely accepted 'confirmatory' test is Western blot. A reaction in a screening assay simply indicates that antibody from the specimen is bound to the solid phase: this can be an HIV-specific or nonspecific reaction. WB provides additional information about specificity, in terms of reactivity with particular HIV proteins. The reaction pattern obtained may be interpreted as positive,

negative or indeterminate according to the application of one of several sets of accepted criteria. The disadvantages of WB are its cost, subjectivity and high rate of indeterminate reactions. Stringent criteria, while achieving high specificity, lead to low sensitivity. Thus, some sera from infected subjects only give indeterminate results. Conversely, low stringency criteria lead to some false-positive diagnosis by WB. A further disadvantage is that criteria have usually been determined using a particular manufacturer's product. When these are applied to a different WB they may not be valid.

Line immunoassays resembling WB and 'dot-immunoblots', each using several synthetic antigens derived from different HIV genes are now being widely used. They are usually more rapid and at least as sensitive as WB. Interpretative criteria vary but are generally similar to WB, with the same three possible outcomes.

NAT–PCR-based Methods

The nucleic acid amplification test–polymerase chain reaction (NAT–PCR)-based assays detect the nucleic acid of HIV directly in an infected patient's sample and is employed as a confirmatory method. The expense and technical demands of PCR will, for the foreseeable future, restrict its use to carefully selected cases and to providing support to investigations of simpler, less expensive confirmatory strategies.

Alternative Approach to Confirmatory Testing

This approach seeks to determine if a sample reaction in a screening test will also give a positive signal in at least two further 'independent' screening assays. Assays were originally defined as 'independent' if they were each of a different type. But this concept has recently been broadened to incorporate assays using antigens from different sources, e.g. viral, recombinant DNA expression products and synthetic peptides. Just as there have to be trials of WB to develop accurate interpretive criteria, particular combinations of assays must be examined to demonstrate that they are generally independent of each other, i.e. they are not susceptible to the same nonspecific effects.

It has recently been shown that a combination of rapid screening assays can also give diagnosis as accurate as WB, and more cheaply. This option should be particularly useful for anti-HIV confirmation especially in the developing world, as a cost effective solution.

WHO/UNAIDs Strategies for HIV Antibody Screening

These three strategies take into account the objective of testing (i.e. whether for transfusion safety, surveillance or diagnosis) and the local seroprevalence rate. Depending on these factors, three different algorithms for repeat and confirmatory testing have been proposed to confirm initial screening results (Fig. 21.19).

They avoid the use of the Western blot for confirmation of positive screening test result; although Western Blot confirmation is still the rule in the USA, Germany and other countries, it can be replaced by more economical testing algorithms using ELISA-type assays and/or rapid/simple test devices.

The successful confirmation using these strategies requires the user to distinguish strictly between assay results (non-reactive or reactive) and patient diagnosis ("Positive" or "Negative").

For all strategies, the first (screening) test (Test 1) should be chosen for its very high sensitivity (>99%) (to avoid false-negative results), while the confirmatory tests (Tests 2 and 3) must have a high specificity (>99%). No test performance characteristic must be <95%. Furthermore, the different tests used for each strategy must employ different formats.

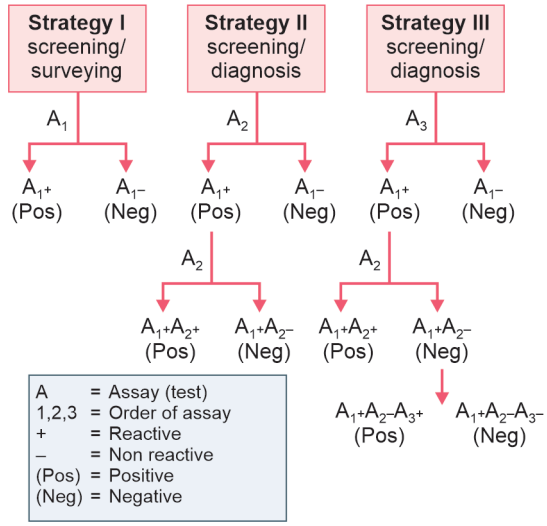


Fig. 21.19: WHO/UNAIDs strategies for HIV antibodies screening

Strategy I requires only one test. It is to be used for:

- Transfusion screening (if no results are to be provided)
- Surveillance (if prevalence >10% and no results are to be provided)
- Diagnosis (only if patient is symptomatic and population HIV prevalence >30%)

NB: This is not normally adequate for diagnosis!

Strategy II requires up to two tests and is to be used for:

- Surveillance (if prevalence <10% and no results are to be provided)
- Diagnosis if patient symptomatic and population prevalence <30%, or if patient asymptomatic and population prevalence >10%.

Strategy III requires up to three tests for:

- Diagnosis if patient asymptomatic and population HIV prevalence <10%.

Discrimination between HIV-1 and HIV-2 Infection

Tests that claim to distinguish HIV-1 from HIV-2 infection cannot necessarily be regarded as confirmatory tests. Some of them rely on a reaction with a single short length oligopeptide and may lack sensitivity for antibody from all individuals infected by the homologous HIV type. However, most of the confirmatory methods already mentioned will discriminate to some degree between HIV-1 and HIV-2. Cross reactions, between env and gag antigens in WB make it less efficient than other approaches such as utilizing a pair of type-specific competitive EIAs or a line immunoassay employing HIV-1 and HIV-2 specific synthetic antigens. Also, the cost of two WB strips or PCR for HIV-1 and HIV-2 is high and it is doubtful whether such expenditure should be committed to making a differential diagnosis for the small proportion of cases in which more simple and inexpensive approaches fail.

Pediatric HIV Infection

Since the first report of an infant with AIDS in November 1982, the incidence of HIV infection in the pediatric population has increased exponentially. It is estimated that HIV pandemic is responsible for the deaths of 3 million women and more than 2.5 million children worldwide. Additionally about 5.5 million children might have been orphaned because of premature death of their parents.

It has been proposed that when maternal infection rates reach 2–3% globally, HIV infection acquired by the perinatal route could become the most common cause of death in children. Of the reported children, younger than 13 years old in the USA, 90% were born to mothers with HIV or HIV risk factors, 5% received blood transfusions or tissue transplants, 3% were hemophiliacs and 1% had no identified risk factor.

Transmission of HIV from Mother to Infants

Transmission of HIV from mother to infants is not absolute but relatively efficient. Prospective studies have reported mother-to-infant HIV transmission in 13–39% of infants born to HIV- infected mothers. Perinatal transmission may occur before, during or after birth. Many characteristics of the HIV infected mothers may be associated with increased risk of perinatal HIV transmission; women with symptomatic HIV infections or AIDS may have an increased risk of transmitting infection to their infants as compared to asymptomatic pregnant mothers.

Higher transmission rates have been reported for infants delivered vaginally than those by cesarean section. In some studies presence of other STDs during pregnancy, such as syphilis, appear to increase the likelihood of HIV transmission possibly due to placentitis associated with *Treponema pallidum* coinfection. Other ulcerative genital infections may also increase the transmission rates.

Perinatal feeding practices can also affect vertical HIV transmission as HIV infection does occur via breastfeeding. Type of milk ingested (colostrum versus later milk) duration of breast-feeding, duration of mother's HIV infection, her viral load and the antibody content in her milk influences mother-to-infant transmission rates.

The working definition of intrauterine infection as suggested by experts is a positive PCR or HIV culture or a p24 antigen test on the infants blood within 48 hours of life. Perinatal infection is defined as negative assays shortly after birth and positive results occurring later. Voluntary testing for HIV to recognize HIV infected mothers can benefit through antiretroviral therapies, early prophylaxis of genital infections, providing passive HIV antibody to pregnant women, fetus and the newborn and providing active or passive enhancement of fetal and neonatal anti-HIV specific immunity.

Diagnostic Challenges: Child Born to a HIV Infected Mother

Diagnosis of HIV infection in children born to HIV infected mothers is complicated by the presence, in almost all cases, of the maternal anti-HIV antibody which crosses the placenta to the fetus. Overall, less than one third of the children are actually infected. Since HIV antibody assays such as EIA, immunoblots primarily detect IgG class of antibodies and in infants, this class of antibody may persist from upto 9–18 months, conventional antibody tests cannot be used to reliably predict the HIV infection in children under 18 months of age. In the first year of life, HIV infection can be reliably diagnosed by PCR and viral culture, since they can correctly identify up to 50% of the infected infants at birth and nearly 100% of the infected infants by 6 months of age. It must be however recognized that false-positive PCR results occur in the first week of life and the standard p24 antigen assay is less sensitive than culture or PCR as it fails to detect immunocomplexed p24 antigen.

The additional potentially useful methods currently under development and evaluation are:

a. Test based on *in vitro* production of virus specific antibodies by patients lymphocytes.
b. Detection of patient specific IgA antibodies to HIV, since the IgA does not cross the placenta.

RAPID IMMUNOCONCENTRATION TEST FOR HIV 1 AND HIV 2 ANTIBODIES FLOW-THROUGH METHOD

Retroquick-HIV®
Courtesy: Tulip Group of Companies

INTRODUCTION

Retroquic-HIV is a membrane based flow through immunoassay for the detection of antibodies to HIV 1 and HIV 2 in human serum and plasma. Highly purified synthetic peptides of gp 120 and gp 41 (HIV 1) and gp 36 (HIV 2) corresponding to the immunodominant regions of the HIV 1 and HIV 2 utilized in the test system assist in visual, qualitative, simultaneous detection and differentiation of antibodies to HIV 1 and 2.

Summary

Acquired immunodeficiency syndrome (AIDS) is caused by at least two retroviruses, the HIV 1 and the HIV 2, collectively referred to as HIV 1/2. Antibodies to HIV 1 envelope protein (gp 120), transmembrane protein (gp 41) and HIV 2 transmembrane protein (gp 36) are prevalent in sera of individuals with AIDS or ARC or who are at high risk of contracting AIDS. Detection of these antibodies indicates exposure to the HIV 1/2 virus.

Principle

Retroquic-HIV test comprises of a test device striped with distinct bands of purified gp 120 and gp 41 synthetic peptide specific to HIV 1 at test region '1' and gp 36 synthetic peptide specific to HIV 2 at test region '2'. The third band striped at region 'C' corresponds to the assay performance control. First the membrane assembly is hydrated with wash buffer and then the specimen is added. Antibodies to HIV 1 and/or 2 if present, are captured by the respective antigens. After washing with wash buffer, Protein A conjugated gold sol reagent is added to reveal the presence/absence of bound antibodies. Post-final wash a positive reaction is visualized by the appearance of purple colored bands at the test region '1' and/or '2'. The absence of bands at test region '1' and '2' is a negative test result. The appearance of control band serves to validate sample addition, reagent and assay performance.

Reagents and Materials Supplied

Kit Components

Retroquic-HIV immunoconcentration test kit for HIV 1 and HIV 2 antibodies comprises the following components:

1. Ready to use individually pouched, flow through test devices striped with HIV 1 specific purified synthetic peptides at test region '1' and HIV 2 specific purified synthetic peptides at test region '2' and a blue dyed protein A based control band at region 'C' along with a specimen dropper and dessicant.
2. Dropper bottle with ready to use wash buffer solution.
3. Dropper bottle with ready to use protein A conjugated gold sol solution.
4. Package insert.

Storage and Stability

The unopened retroquic-HIV kit, as well as kit components upon opening, must be stored at 2–8°C, till the duration of the shelf life as indicated on the kit/kit component labels:

1. *In vitro* diagnostic test. Not for medicinal use.
2. Read package insert carefully before performing the test.
3. Do not use beyond expiry date.
4. Flow-through device, wash buffer and protein A conjugate of the same lot are optimized as a system. It is important that the kit components of the same lot are used for achieving accurate and reproducible results. Do not intermix reagents from different lots.
5. The sequence of addition of reagents should be followed meticulously for achieving accurate results.
6. Handle all specimens as potentially infectious.
7. Follow standard biosafety guidelines for personal protection, handling and disposal of potentially infectious material.
8. After use, the kit components must be returned to the recommended storage temperature immediately.

Specimen Collection and Preparations

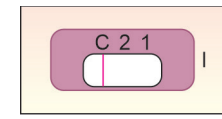
1. No prior preparation of the patient is required before sample collection by approved techniques.
2. Fresh serum/plasma is preferable. Serum/plasma may be stored at 2–8°C up to 24 hours in case of delay in testing. For long-term storage, freeze the specimen at –20°C.
3. Repeated freezing and thawing of the specimen should be avoided.
4. Do not use hemolyzed, clotted, contaminated, viscous/turbid specimen.
5. Specimen containing precipitates or particulate matter must be centrifuged and the clear supernatant only used for testing.
6. Do not heat-inactivate the specimen.
7. Frozen samples for retrospective studies must be centrifuged at 3000 rpm for 15 minutes and the clear supernatant must be used for tests.

Test Procedure

1. Bring all reagents and specimen to room temperature (25–30°C) before use. Tighten the Wash Buffer solution and Protein A Gold Conjugate dropper bottle caps in a clockwise direction to pierce the respective dropper bottle nozzles. The addition of specimen/reagents must be done at the centre of the reaction port, holding the sample dropper/dropper bottles in a vertical position. Ensure the drops are free falling. Use a new sample dropper for each specimen to avoid cross contamination.
2. Tear open the foil pouches and retrieve the required number of Retroquic-HIV membrane test devices and label appropriately.
3. Add two drops of wash buffer into the reaction port of the device and allow to soak through completely.
4. Using the sample dropper provided, add one drop of the serum/plasma specimen into the reaction port. Allow to soak through completely.
5. Add three drops of wash buffer to the reaction port and allow to soak through completely.
6. Add two drops of protein A gold conjugate to the reaction port and allow to soak through completely.
7. Add two drops of wash buffer and allow the wash buffer to soak through completely.
8. Read and record the results immediately.

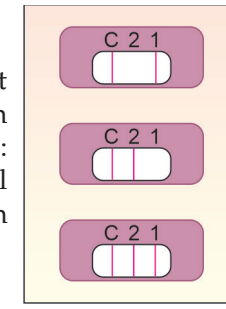
Interpretation of Results

Negative Test Result



Appearance of only one control band corresponding to control region 'C'.

Positive Test Results



In addition to the control band 'C', appearance of reactive band at test region '1': Specimen positive for antibodies to HIV 1. In addition to the control band 'C', appearance of reactive band at test region '2': Specimen positive for antibodies to HIV 2. In addition to the control band 'C', appearance of reactive bands at test region '1' and test region '2' Specimen positive for antibodies to HIV 1 and HIV 2.

Invalid Test Result

The test should be considered invalid if neither the test band nor the control band appears. In case of invalid results, the test should be repeated using a fresh device.

Remarks

1. The addition of reagents must be accomplished without interruptions.
2. After addition of the wash buffer, in step 7 of the procedure, if the background in the reaction port is high, the samples must be recentrifuged appropriately so as to pellet invisible particulate matter. Test should be rerun with the clear supernatant.
3. The presence of antibodies to HIV 1/2 indicates previous exposure to HIV 1 and/or HIV 2 virus but does not constitute a diagnosis of AIDS.
4. Absence of antibodies to HIV 1/2 does not indicate that an individual is absolutely free of HIV 1 or HIV 2 as the collection of sample and its timing vis-a-vis seroconversion will influence the test outcome.
5. Since HIV 1 and HIV 2 viruses are similar in genomic structure and morphology and antibodies to them have (30–70%) cross reactivity, reactive test bands for HIV 1 and HIV 2 do not necessarily imply mixed infection with HIV 1 and HIV 2.
6. Though Retroquic-HIV is a reliable and sensitive screening test, it should not be used as a sole criterion for diagnosis of HIV infection.
7. All positive specimen should be further tested using appropriate supplemental confirmatory tests.
8. As in all tests the results must be correlated with clinical findings before arriving at the final diagnosis.
9. Since various tests for HIV 1/2 differ in their performance characteristics and antigenic composition, the reactivity patterns may differ.
10. The results of Retroquic-HIV must be read within 30 minutes of test completion.
11. Do not compare the intensity of the test lines and the control lines to judge the concentration of the antibodies in the test sample.
12. Testing of pooled specimen is not recommended.
13. The control band in fresh unused membrane test devices is blue colored and changes to blackish purple color after test performance.
14. The control band would not develop if the sample addition has not been done.

THIRD GENERATION DOUBLE ANTIGEN SANDWICH ENZYME LINKED IMMUNO-SORBENT® ASSAY (ELISA) FOR THE DETECTION OF TOTAL ANTIBODIES TO HIV-1 AND HIV-2 INCLUDING HIV-1 GROUP "O" IN HUMAN SERUM OR PLASMA

Retrolisa 3.0®

Courtesy: Tulip Group of Companies

Retrolisa 3.0 is intended to be used for the detection of total antibodies (i.e. IgG, IgM, IgA etc) to both HIV-1 and HIV-2 including HIV-1 group "O" in human serum or plasma.

Summary and Explanation

Present evidence indicates that acquired immunodeficiency syndrome is caused by HIV- 1 and HIV-2. The viruses are transmitted by sexual contact, exposure to blood (including sharing contaminated needles and syringes) or certain blood products, or transmitted from an infected mother to her fetus or child during the prenatal period. Presence of antibodies to the virus in the serum of a patient indicates viral infection.

Retrolisa 3.0 is a third generation solid phase enzyme-linked immunosorbent assay (ELISA) which employs highly purified recombinant antigens representing envelope glycoprotein gp41 and core protein p24-O subtype fusion polypeptide of HIV-1 and envelope glycoprotein gp36 of HIV-2. The use of HIV antigens to sandwich specific antibodies enables detection of IgG, IgM and IgA antibodies thus reducing window period and earlier detection of seroconverters. Some reports suggest that third generation ELISAs like RETROLISA 3.0 can detect HIV antibodies at 2–3 weeks earlier than second gereration ELISAs that detect only IgG.

Principle of the Assay

Microwell strips are coated with recombinant antigens, gp41, p24-O subtype fusion polypeptide and gp36 representing both HIV-1 and HIV-2. Samples along with positive and negative controls are added in the coated wells and incubated. The wells are washed to remove unbound components. The presence of bound antibodies is detected by adding antigen-HRP conjugate. After washing wells to remove unbound enzyme, substrate is added. The reaction is stopped after specified time with acid and absorbance is determined for each well at 450 nm with an ELISA reader. The cutoff value is calculated by the given formula and absorbance of all the wells are compared with the cutoff value. Any sample having absorbance more than the cutoff value is considered reactive.

Kit Components

Retrolisa 3.0 has following components:

1. Coated microwells: Microwells coated with recombinant antigens representing both HIV-1 and HIV-2. Ready to use.
   96 wells: (3 × 8) × 4 pouches
   192 wells: (3 × 8) × 8 pouches
   480 wells: (3 × 8) × 20 pouches
2. Positive control: Inactivated and stabilized human serum reactive for HIV-1 with preservatives.
3. Negative control: Inactivated and stabilized human serum nonreactive for HIV-1 and HIV-2, HBsAg and HCV.
4. Conjugate: Antigen-HRP conjugate (50X). To be diluted 50 times with conjugate diluent.
5. Conjugate diluent: Buffered solution containing stabilizing proteins and preservatives.

6. Sample diluent: Buffered solution containing stabilizing proteins and preservatives.
7. Substrate: Solution containing tetramethyl benzidine (TMB) and hydrogen peroxide. Ready to use.
8. Wash buffer: Buffer containing surfactants (20X). To be diluted 20 times with distilled or deionized water.
9. Stop solution: Diluted sulfuric acid.
10. Microwell holder.
11. Instruction for use.
12. ELISA protocol sheet.
13. Plate sealer.

Storage and Stability

1. Retrolisa 3.0 kit is stable at 2–8°C up to the expiry date printed on the label.
2. Coated microwells should be used within two months of opening the pouch. Once opened, the pouch must be sealed properly to protect from moisture.
3. Diluted conjugate must be used immediately.
4. Diluted wash buffer is stable up to one week.

Material Required But not Provided

1. Manual or automotive pipette
2. Pipettor tips
3. Incubator
4. Absorbent sheets
5. ELISA washer
6. ELISA reader
7. Pipetting troughs or boats
8. Disinfectant
9. Reagent grade water
10. Disposable gloves
11. Timer
12. Biohazard waste container
13. Serological pipettes.

Sample Collection

1. No prior preparation of the patients is required.
2. Collect blood specimen by venipuncture according to the standard procedure.
3. Serum or plasma can be used.
4. Specimen should be free of particulate matter and microbial contamination.
5. Preferably use fresh sample. However, specimen can be stored refrigerated for short duration. For long storage, freeze at –20°C or below. Do not freeze samples in frost-free freezer.
6. Specimen should not be frozen and thawed repeatedly.
7. Do not heat inactivate before use.
8. Specimen containing precipitate or particulate matter should be clarified by centrifugation prior to use.

Precautions

1. Bring all reagents and specimen to room temperature before use.
2. Do not pipette any material by mouth.

3. Do not eat, drink or smoke in the area where testing is done.
4. Use protective clothing and wear gloves when handling samples.
5. Use absorbent sheet to cover the working area.
6. Immediately clean up any spills with sodium hypochlorite.
7. Dispose off all the reagents and material used as if they contain infectious agent.
8. Neutralize acid containing waste before adding hypochlorite.
9. Do not use kit after the expiration date.
10. Do not mix components of one kit with another.
11. Always use new tip for each specimen and reagent.
12. Do not let the dispensing tip of ELISA washer liquid in the wells.
13. Do not allow liquid from one well to mix with other wells.
14. Do not let the strips dry in between steps.
15. Incubation times and temperatures other than those specified may give erroneous results.
16. Cross contamination of reagents or samples may give false results. Samples must remain confined to microwells during testing.
17. Inadequate removal of residual wash buffer can cause inconsistent color development. Microwell strips should be tapped vigorously and blotted on absorbent paper or towels to minimize residual wash buffer.
18. Reusable glassware must be washed and thoroughly rinsed free of detergents prior to use. All glassware must be clean and dry before use.
19. Adequate washing of wells is extremely important. Inadequately washed wells will exhibit high background values, and may show false positive values. For manual washing, aspirate the contents of the wells, then fill each well with wash buffer solution. Avoid cross-contamination of the wells, particularly in the first wash after aspiration. Drain all of the wash buffer from the wells by inverting, then shaking residual wash buffer from the wells with a sharp "snapping" motion of the wrist. Repeat these steps for at least six washes. The wells should then be tapped vigorously on a paper towel or other absorbent material to remove all traces of residual wash buffer. The use of an automated microwell washing system will assure consistent washing of the wells, and is recommended.

Reagent Preparation

1. Dilute wash buffer 20 times (for example add 5 mL concentrated buffer to 95 mL distilled or deionized water).
2. Dilute conjugate 51 times (for example add 20 µL concentrated conjugate to 1 µL conjugate diluent).

Test Procedure

1. Bring all the reagents and specimen to room temperature before use.
2. Take out required number of strips and immediately close the pouch.
3. Prepare data sheet indicating the location of controls and specimen.
4. Use controls in duplicate.
5. Add 100 µL sample diluent in each wells.
6. Add 5 µL of control or specimen in separate wells.
7. Apply plate sealer and incubate for 30 minutes at 37°C.
8. Wash each well by filling approximately 350 µL diluted wash buffer, giving 30 seconds soak time for each wash and aspirating/flicking off six times. Blot dry.

No. of strips	1	2	4	6	8	10	12
50 × conjugate	20 µL	40 µL	80 µL	100 µL	140 µL	180 µL	200 µL
Conjugate diluent	960 µL	1960 µL	3920 µL	4900 µL	6860 µL	8820 µL	9800 µL

9. Add 100 µL diluted conjugate in each well and incubate for 30 minutes at room temperature (20–28°C).
10. Wash six times as in step 8. Blot dry.
11. Add 100 µL substrate in each well and incubate at room temperature (20–28°C) away from light for 30 minutes.
12. Stop reaction by adding 100 µL stop solution. The stop solution should be added in the same sequence as substrate addition.
13. Read the absorbance at 450 nm with 600–700 nm as reference with in 30 minutes of stopping the reaction.

Run Criteria

1. The individual absorbance value of negative controls should be less than 0.1.
2. The individual absorbance value of positive controls should be more than 1.0.

Calculations

The cutoff value (COV) is calculated by adding 0.2 to average absorbance value of negative control.

Example:

COV = Av.Nc + 0.2			
Absorbance of negative control		*Absorbance of positive control*	
1	0.02	1	2.723
2	0.015	2	2.703
Avg NC	0.017	Avg PC	2.713

Cutoff value = Av.Nc + 0.2 = 0.017 + 0.2 = 0.217

Sample Data				
Well	*Absorbance*	*Mean*	*Cutoff*	*Result*
NC	0.02			
NC	0.015	0.017		
PC	2.723			
PC	2.703	2.713	0.217	
Sample 1	3.487			Reactive
Sample 2	0.654			Reactive
Sample 3	0.056			Nonreactive

Note

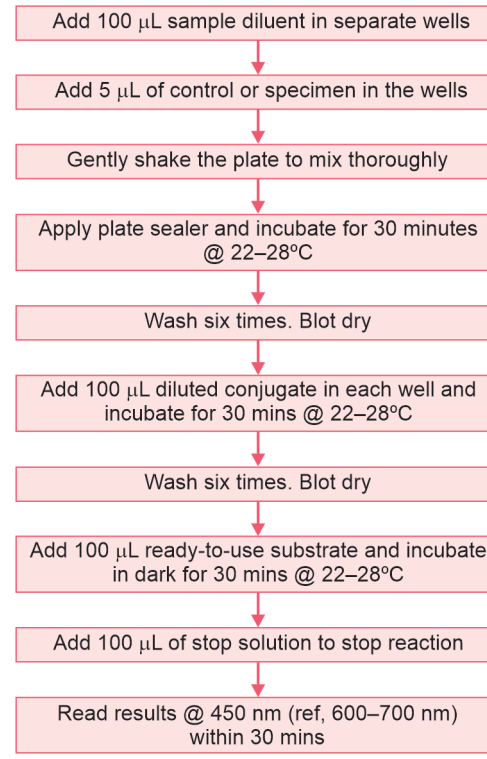
All ELISA readers have a linear measuring range (approx. 2.5A). Beyond this range the OD values are nonlinear. That is why some instruments can be preprogrammed to show "out of range" values if the OD exceeds 2.5 or 3.00 Retrolisa 3.0 positive control may show "out of range" values in such instruments even after dilution. Please note that this

indicates a valid run (provided the negative control value is <0.1) and does not interfere with assay results.

Interpretation of Results

1. Samples with absorbance value less than the cutoff value are considered non-reactive by Retrolisa 3.0 ELISA kit and are considered negative for HIV 1/2 antibodies.
2. Samples with absorbance value equal to or greater than cutoff value are considered reactive by Retrolisa 3.0 ELISA kit. The original sample should be retested in duplicate. Initially reactive sample that do not react in either of duplicate are considered negative for antibodies to HIV 1/2. Initially reactive sample that reacts in either or both duplicates are considered repeatedly reactive.
3. If a sample is repeatedly reactive the probability of antibodies to HIV 1/2 are high, especially with patients at high risk or high absorbance values. Such samples should be retested with supplemental third generation tests. Specimens that are repeatedly reactive in ELISA but not reactive in additional testing are considered indeterminate (Flowchart 21.1).

Flowchart 21.1: Retrolisa 3.0 procedure


Add 100 μL sample diluent in separate wells
Add 5 μL of control or specimen in the wells
Gently shake the plate to mix thoroughly
Apply plate sealer and incubate for 30 minutes @ 22–28°C
Wash six times. Blot dry
Add 100 μL diluted conjugate in each well and incubate for 30 mins @ 22–28°C
Wash six times. Blot dry
Add 100 μL ready-to-use substrate and incubate in dark for 30 mins @ 22–28°C
Add 100 μL of stop solution to stop reaction
Read results @ 450 nm (ref, 600–700 nm) within 30 mins

Remarks

1. Though Retrolisa 3.0 is a reliable screening assay, it should not be used as a sole criterion for diagnosis of HIV infection.
2. Absence of antibodies to HIV does not indicate that an individual is absolutely free of HIV infection as the collection of sample and its timing vis-à-vis seroconversion will influence the test outcome.
3. Since various tests for HIV differ in their performance characteristics and antigenic composition, their reactivity patterns may differ.
4. Testing of pooled samples is not recommended.
5. As with all diagnostic tests, a definitive clinical diagnosis should not be based on the result of a single test, but should only be made by the physician after all clinical and laboratory findings have been evaluated.
6. Retrolisa 3.0 should only be used as a screening test and its results should be confirmed by other supplemental method before taking clinical decisions.

CHAPTER

22

Bone Marrow in Nonhemopoietic Disease

The bone marrow may be involved in a number of disorders which are not primary diseases of the hemopoietic system. Aspirates or trephine biopsies may be used to assess bone marrow involvement after primary diagnosis based on material from other sites, or may be responsible for the primary diagnosis itself.

METASTATIC BONE TUMORS (Fig. 22.1)

The most common malignant tumors of bone are metastatic deposits from primary sites elsewhere in the body. Carcinomas of the breast, lung, kidney, thyroid and prostate are very likely to involve the marrow; stomach, pancreas, colon and rectum tumors involve the marrow less frequently but virtually any malignant tumor may reach the bone and the marrow therein.

In advanced disease, leukoerythroblastic changes may be found in the blood film: there may be evidence of bone marrow failure. The skeletal lesions are predominantly osteolytic, presenting as radiolucent areas or radiographic examination.

Extensive osteoclastic resorption of bone is often found in trephine biopsies. Almost all tumors provoke some bone healing and sometimes new bone deposition: osteoblastic activity is most pronounced in metastatic spread from carcinoma of the prostate.

Occasionally, fragments of epidermis are carried into the marrow cavity during trephine biopsy, but the well-differentiated nature of such fragments allows easy differentiation from metastatic carcinoma.

Involvement of bone marrow by tumors other than carcinomas also occurs, e.g. by melanoma, neuroblastoma and medulloblastoma. Bone marrow involvement due to

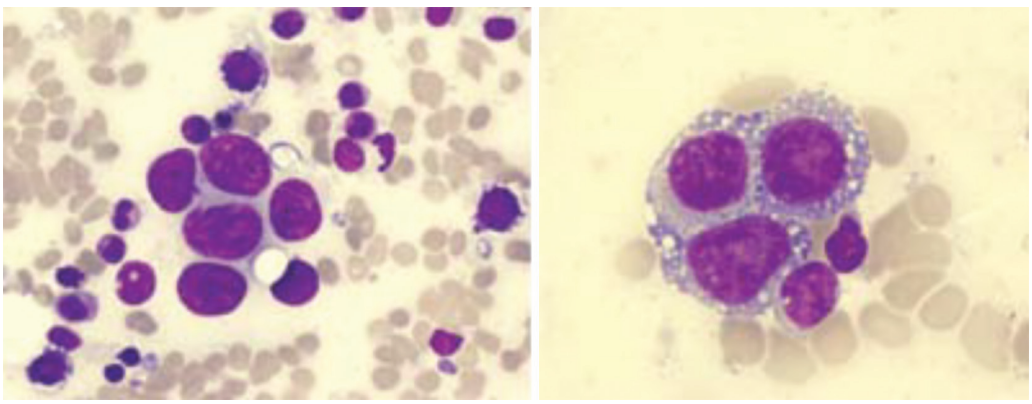


Fig. 22.1: Metastatic carcinoma to bone marrow

malignant lymphomas and histocytic proliferative disorders has already been discussed earlier. Although majority of primary bone tumors do not spread to parts of the skeleton but Ewing's sarcoma may sometimes metastasize to distant bones.

Metastatic tumors in the marrow may be detected using monoclonal antibodies and indirect immunofluorescence or alkaline techniques. Anti-milk fat globulin may detect breast carcinoma: anticytokeratin may detect epithelial carcinoma: and antidesmin (intermediate filaments) may detect mesenchymal tumors.

MAST CELL DISEASE (Fig. 22.2)

Systemic mastocytosis is a rare disease of adults in which a persistent and progressive cutaneous eruption is a characteristic presentation. Radiography reveals usually multiple, regularly rounded, lytic lesions or new bone formation in the skeleton. In severe cases there may be lymphadenopathy, hepatosplenomegaly and extensive infiltration of the bone marrow by mast cells. Evidence of disease may also be found in splenectomy. Disease confined to the skin and bone is frequently associated with prolonged survival; but patients who develop extensive reticuloendothelial involvement often die soon after diagnosis.

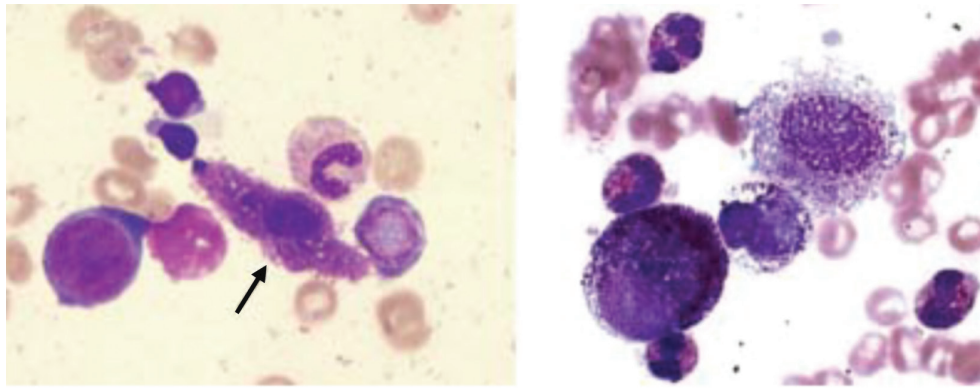


Fig. 22.2: Mast cells in the marrow

GRANULOMATOUS DISEASE

TUBERCULOSIS (Fig. 22.3)

Hematogenous spread of tubercle bacilli may bring them to bone marrow; aspirates from patients with suspected miliary or other typical hematogenous form of tuberculosis may show characteristic epithelioid granulomas. If left untreated, the disease becomes extensive, particularly in the anterior aspects of the vertebral bodies and in the metaphyseal areas of long bones. The tubercular foci may progress, producing cystic areas of osteomyelitis which erode the end plates and involve the nearby joint spaces. Tuberculous spondylitis (Pott's disease) involves the anterior aspects of vertebral bodies with subsequent wedging and eventual collapse.

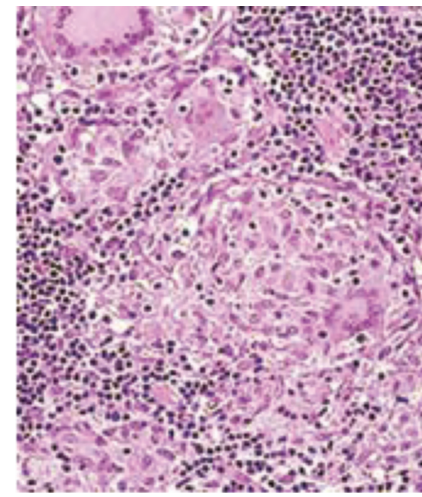


Fig. 22.3: Granuloma in the marrow

SARCOIDOSIS

Of unknown etiology, this granulomatous disorder most frequently affects the middle-aged. It is characterized by widespread epithelioid cell granulomas, depression of delayed hypersensitivity and lymphoproliferation. Multisystem involvement is characteristic with intrathoracic disease affecting 90% of patients, occular and stem involvement in about 25% each, and erythema nodosum in up to a third of cases. Evidence of disease may be found during bone marrow examination or at splenectomy.

OTHER GRANULOMAS

Brucellosis or foreign body granulomas may be found rarely during routine examination of trephine biopsies.

KALA-AZAR (VISCERAL LEISHMANIASIS) (Fig. 22.4)

Common in eastern Uttar Pradesh and Bihar. The causal organism, *Leishmania donovani*, is transmitted by the bite of sandflies of the genus.

Phlebotomus: The nonflagellated amastigote forms of the organism are distributed widely through reticuloendothelial macrophages in the bone marrow: spleen and liver. Diagnosis is usually made by examining bone marrow splenic aspirates or biopsy specimens. Clinical features include prolonged fever, lassitude, weight loss, splenomegaly, hepatomegaly, anemia, leukopenia and polyclonal increases in immunoglobulin.

OTHER INFECTIONS

Bone marrow examination has little significance in the diagnosis and management of osteomyelitis although, occasionally, initial evidence of a disseminated fungal infection may be uncovered. The bone marrow and spleen may be involved in cases of disseminated histoplasmosis. The *Histoplasma capsulatum* fungal organism may be seen inside macrophages in stained aspirates.

GAUCHER'S DISEASE (Fig. 22.5)

This relatively common familial disorder is characterized by the accumulation of glucocerebrosides (especially glycosyl-ceramide) in reticuloendothelial cells, which occurs because the enzyme β-glucocerebrosidase (glycosidase) is deficient. Three types occur: (i) chronic adult, (ii) acute infantile neuropathic, and (iii) subacute neuropathic with

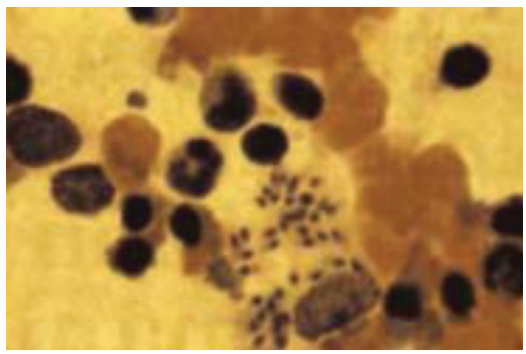


Fig. 22.4: Leishmania bodies in the marrow

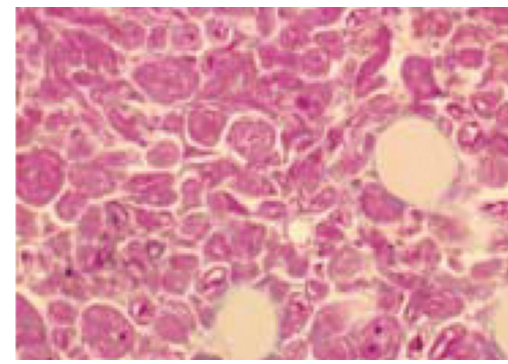


Fig. 22.5: Gaucher's cells in the bone marrow

onset in childhood or adolescence. The chronic adult non-neuropathic form of disease is accompanied by hepatosplenomegaly and bone lesions, and sometimes lymphadenopathy, skin pigmentation and pingueculae. The most acute neuropathic forms present in infancy and survival beyond the first three years of life is rare. A juvenile form may present in childhood with features of the chronic adult form as well as progressive neurological dysfunction.

A presumptive diagnosis of Gaucher's disease may be made when Gaucher's cells are detected in marrow aspirates and trephine biopsies. Diagnosis can be confirmed by demonstration of absence or severe deficiency of the enzyme glucosyl ceramide β-glucosidase in fibroblast cultures. Gaucher's cells are also found in the liver and spleen. Most patients with this condition have elevated plasma acid phosphatase activity (which is not inhibited by L-tartarate). Over 50% of adult patients usually have asymptomatic radiographic changes, such as cortical expansion of the characteristic radiolucent area.

NIEMANN-PICK DISEASE (Fig. 22.6)

Niemann-Pick disease is a sphingomyelin lipidosis, rarer then Gaucher's disease, and characterized by extensive tissue storage of sphingomyelin, hepatic and splenic enlargement, and large lipid-filled macrophages in the bone inarrow. In its best defined forms there is an inherited deficiency of the enzyme sphingomyelinase, and sphingomyelin concentration in the tissues is up to a hundred times higher than normal. As is Gaucher's disease, there are acute neuropathic and chronic non-neuropathic forms.

The disease is suspected in young children with hepatosplenomegaly when bone marrow aspirates show the presence of foam cells. Confirmation is by showing low levels of sphingomyelinase in fibroblast cultured from strain of bone marrow. In less severe adult forms of the disease, large numbers of sea blue histiocytes may be found in bone marrow aspirates in addition to classic foam cells.

SEA-BLUE HISTIOCYTE SYNDROME (Fig. 22.7)

Patients with the rare syndrome usually present with splenomegaly and thrombocytopenic purpura. In some cases there is associated hepatic cirrhosis. The inheritance pattern is autosomal recessive. Bone marrow aspirates contain large numbers of 'sea-blue' histiocytes. There is tissue accumulation of phospholipids and sphingomyelin and reduced levels of cellular sphingomyelinase activity have been reported. It appears that the syndrome is a variant of Niemann–Pick disease.

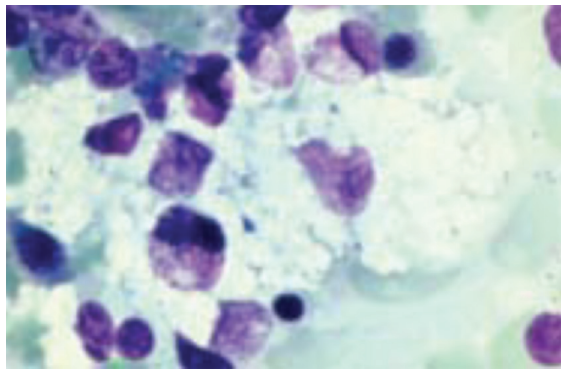


Fig. 22.6: Niemann–Pick disease, marrow

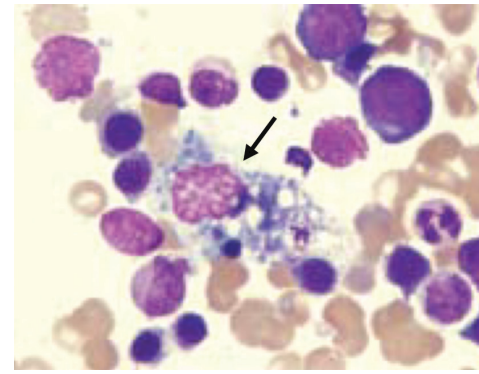


Fig. 22.7: Sea-blue histiocyte disease, bone marrow

CAUSES OF SEA-BLUE HISTIOCYTES

Large Numbers

- Sea-blue histocyte syndrome
- Niemann–Pick disease

Occasional or Moderate Numbers

- Hyperlipoproteinemia
- Hereditary acyltransferase deficiency
- Wolman's disease
- Other lipid storage disorders
- Chronic myeloid leukemia
- Polycythemia vera
- Chronic immune thrombocytopenia (ITP)
- Thalassemia
- Sickle cell disease
- Sarcoidosis
- Chronic granulomatous disease.

CYSTINOSIS

In this recessively inherited disease there is deposition of cystine crystals in the reticuloendothelial and corneal tissues. In its more severe form (cystinosis with Fanconi's syndrome or the de Tori–Fanconi–Lignac syndrome) there is progressive renal degeneration which is fatal during early childhood. Children with this syndrome usually present with anorexia, thirst, polyuria, failure to thrive, rickets or photophobia. Laboratory tests reveal glycosuria, proteinuria, low serum bicarbonate, hypokalemia or hypophosphatemia. The diagnosis is based upon demonstration of cystine crystals in macrophages in bone marrow aspirates.

OSTEOPETROSIS (ALBERS-SCHÖNBERG'S DISEASE OR MARBLE BONE DISEASE)

This rare familial disorder is characterized by an increase in density of all bones due to a functional defect in osteoclasts, with failure of bone resorption and remodeling. Severe forms of the disease present in infancy with anemia and hepatosplenomegaly. These children have little or no bone marrow, hemopoiesis is chiefly extramedullary and blood transfusions are required to sustain life. Peripheral blood shows leukoerythroblastic changes.

Failure to resorb bone results in optic atrophy, deafness and hydrocephalus. Milder cases may present in later childhood or in adult life with retarded growth, anemia and splenomegaly.

AMYLOIDOSIS

CLASSIFICATION OF AMYLOIDOSIS

In some patients, particularly those with reactive systemic amyloidosis, a trephine bone marrow biopsy may reveal the first evidence of amyloidosis.

Type	*Chemical nature*	*Organs involved*
lmmunocyte-related myeloma	Ig light chains and/or parts or their variable regions (AL)	Tongue, skin, heart, nerves, connective tissue, kidneys, liver, spleen
Waldenstrom's macroglobulinemia, heavy chain disease, etc.		
Primary amyloidosis, Reactive systemic Rheumatoid arthritis Tuberculosis Bronchiectasis Hodgkin's disease Mediterranean fever, etc. also familial	Protein A (acute reactive; AA)	Liver, spleen, kidneys, bone marrow
Localized Tumors old age (Aizhelmer's disease) Also in skin	Hormones, protein A with other constituents	Around endocrine tumors, skin, CNS.

RENAL OSTEODYSTROPHY AND OSTEOMALACIA

In uremia, there is resistance to the action of vitamin D and a compensatory parathyroid hyperplasia. Characteristic changes are found in bony architecture on trephine biopsy examination. In mild disease the lesions are predominantly osteomalacic. Microscopically, the trabeculae are increased in thickness and number, and the osteoid seams have defective mineralization. A similar picture is seen in dietary vitamin D deficiency. In severe disease there is also evidence of osteitis fibrosa.

PAGET'S DISEASE OF BONE (OSTEITIS DEFORMANS)

In this disease of unknown etiology. There Is rapid bone formation and resorption in the involved regions of the skeleton. The lesions are essentially local and asymmetrical in early stages and frequently involve the weight-bearing bones especially the sacrum and pelvis. Unsuspected disease may be found during trephine bone marrow biopsy examination. The plasma calcium and phosphorus levels are usually normal, while the alkaline phosphatase level is invariably high. Where there is extremely rapid absorption, areas of fibrosis and intensive osteoclastic activity may resemble the microscopical appearance of osteitis fibrosa.

ANOREXIA NERVOSA

These patients suffer from severe deficiency of carbohydrates, fats and calories but little protein deficiency. The peripheral blood may reveal mild anemia and thrombocytopenia with acanthocytes; the marrow is hypocellular, with fat cells replaced by acid mucopolysaccharides appearing as pink-staining extracellular material.

PRIMARY OXALURIA

In this fatal autosomal recessive metabolic disorder, there is widespread deposition of calcium oxalate crystals in the kidneys and elsewhere in the body. Including the liver, spleen and bone marrow. A number of different enzyme deficiencies have been implicated as causal factors.

CHAPTER

23

Hemoparasites

MALARIAL PARASITES OF MAN

Table 23.1: Morphology of different erythrocytic forms of plasmodia of man

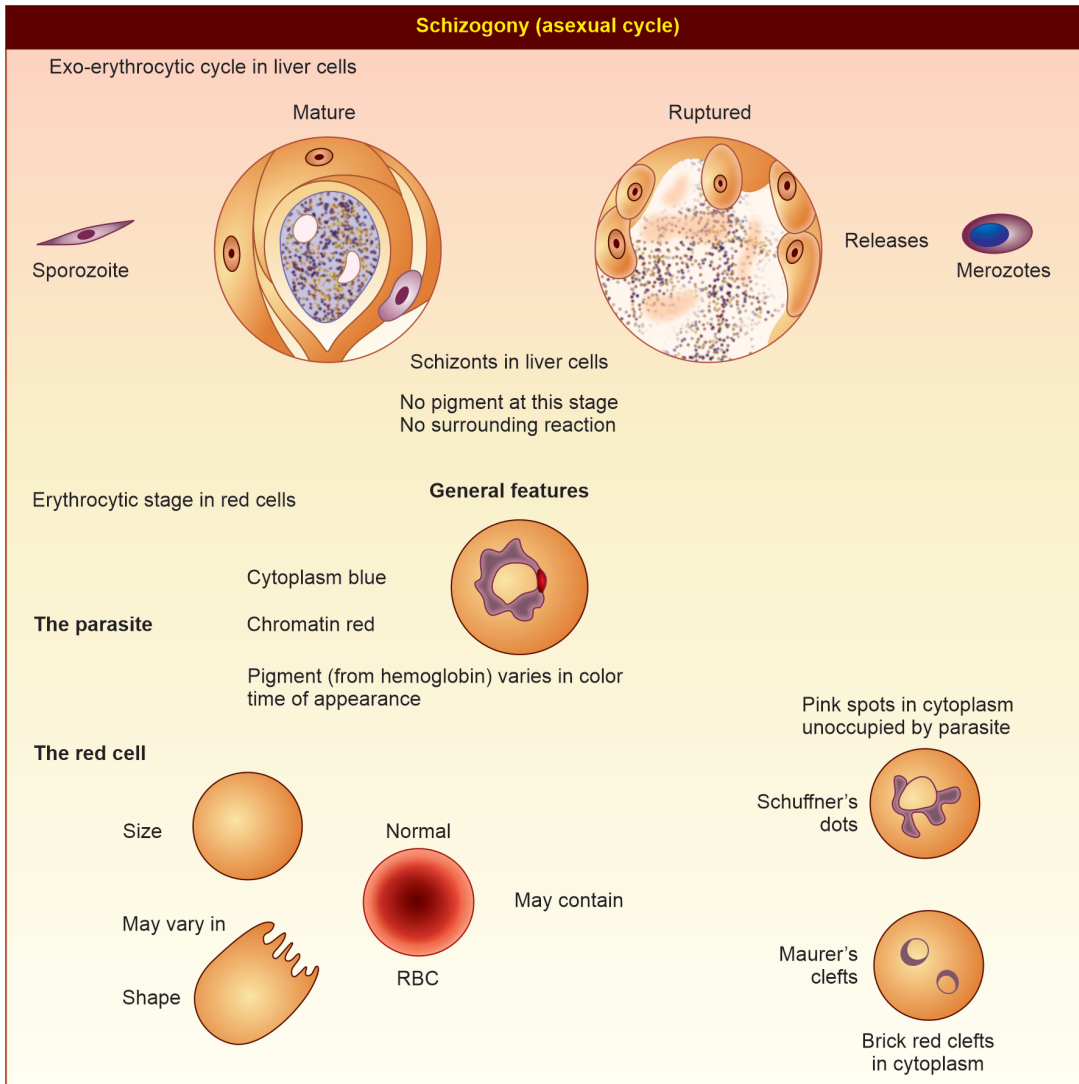
	P. vivax	*P. falciparum*	*P. malariae*	*P. ovale*
Trophozoite				
Size	Relatively large, 2.5 μ	Small; <1.5 μ	Relatively large, 2.5 μ	Relatively large, 2.5 μ
Shape	Round or oval, delicate ring	Round or oval, very delicate ring	Round or oval, compact ring	Round or oval, dense ring
Chromatin	Prominent dot in thin part of cytoplasm or in vacuole; at times two dots	Fine dot; frequently two or bar shaped	A prominent mass often inside the vacuole of the ring. 'Bird' eye form is common	A dense well-defined mass at the thin segment of the cytoplasm
Accole form	At times	Frequent	None	None
Cytoplasm	Thickened opposite to chromatin	No thickening opposite to chromatin	Thicekned all through, more opposite to chromatin	Thickened opposite to chromatin
Pigment	Nil	Nil	May be present	Nil
Number in an RBC	One	May be more	One	One
Growing form rarely seen in peripheral blood				
Size	Large	Small	Small	Small
Shape	Irregular; ameboid with fine streaming cytoplasmic pseudopodia	Compact	Compact; cytoplasm more collected together; Egg form, equatorial band form, ribbon, comet form	Compact; may be slightly ameboid
Vacuole	Prominent	Inconspicuous	Disappears early	Inconspicuous
Chromatin	Dots or threads	Dots or threads; chromatin is relatively more compared to cytoplasm	Dots or threads	Large irregular clumps
Pigment color	Yellowish brown	Black or pepper-like	Dark brown	Dark yellowish brown

Contd.

Table 23.1: Morphology of different erythrocytic forms of plasmodia of man

	P. vivax	*P. falciparum*	*P. malariae*	*P. ovale*
Microgametocyte (male)				
Size	Large (10–12 μ); fills enlarged RBC	Large (8–10 μ × 2–3 μ); larger than RBC	Smaller than size of a normal RBC	About the size of normal RBC
Shape	Round or oval, compact	Kidney or bean shaped; ends bluntly rounded	Round; compact	Round; compact
Cytoplasm	Light blue	Pinkish blue	Reddish blue (stains badly)	Pale blue
Chromatin	Fibril in skin; large, diffuse; stains poorly; lies across equator, surrounding area unstained	Fine granules; scattered through 1/3 of the body of the parasite lie amongst pigment granules, stains lightly in the central part	Fibril in skin; medium, diffuse; arranged in zone like bands, surrounding area unstained	As in *P. vivax*
Pigment	(i) Fine granular (ii) Light brown to yellow brown (iii) Scattered throughout cytoplasm	(i) Fine granular (ii) Blackish (iii) Scattered throughout	(i) Coarse granular (ii) Dark brown (iii) Scattered and also aggregated in chunks and masses	
Macrogametocyte (female)				
Size	Large (12–14 μ; larger than male); fills enlarged RBC	Larger (10–12 μ × 2–3 μ) larger than male; larger than RBC	Smaller than size of RBC	Size of RBC
Shape	Round or oval	Crescent shaped; ends sharply rounded or pointed	Round; compact	Round; compact
Cytoplasm	Deep blue	Deep blue	Deep blue	Deep blue
Chromatin	Condensed into compact mass; eccentric in position often surrounded by a halo	Condensed into a small deep staining compact mass; in position lies in the midst of pigment (no halo)	As in *P. vivax*	As in *P. vivax*
Pigment	(i) Yellow brown (ii) Aggregated in small masses (iii) Arranged at periphery or wreath-like	(i) Black (ii) Granular or small clumps (iii) Arranged round the chromatin masses	(i) Dark brown (ii) and as in *P. vivax;* abundant pigment—pigmented parasite	(i) Dark yellow brown; (ii) and as in *P. vivax*

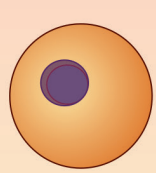
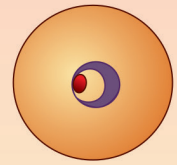
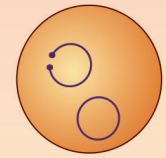
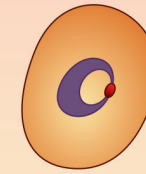
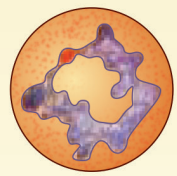
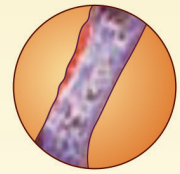
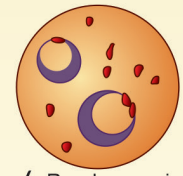
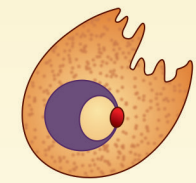
Morphology of Malarial Parasites Stained by Leishman of Giemsa



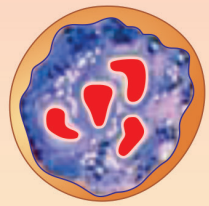
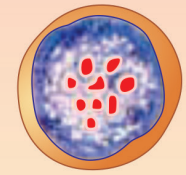
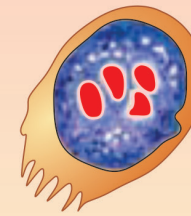
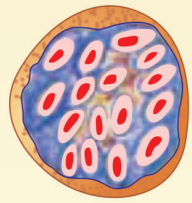
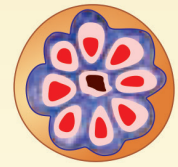
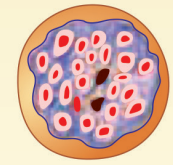
	P. vivax	*P. malariae*	*P. falciparum*	*P. ovale*
Size	Enlarged	Not enlarged	Not enlarged	Slightly enlarged
Color	Pale	Normal	Normal	Pale
Shape	Round	Round	Round (may be crenaled)	Oval may be fimbriated
Schuffner's dots	Present	None	None	Conspicuous
Maurer's clefts	None	None	May be present	None

Morphology of Malarial Parasites Stages in Thin Films

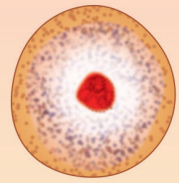
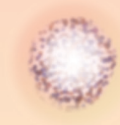
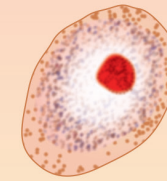
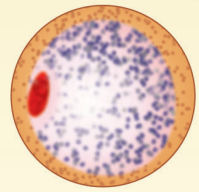
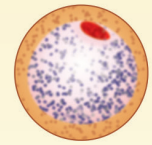
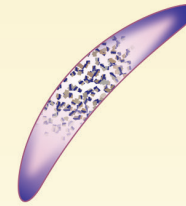
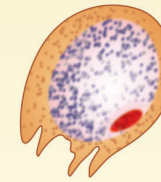
Morphology of material parasites
Stage in thin films

	P. vivax	*P. malariae*	*P. falciparum*	*P. ovale*
Ring forms (early trophozoites)				
				
Size	Up to 1/3rd red cell	Up to 1/3rd	Up to 1/5th	Up to 1/3rd red cell
Shape	Delicate ring	Compact ring	Very delicate ring	Delicate ring
Chromatin	Fine dot sometimes two	One mass others inside ring	Fine dots frequently two	Dense, well defined mass
Accole forms	Sometimes	None	Frequent	None
Pigment	None at this stage	May be present	None at this stage	None at this stage
Developing trophozoites				
			 (Rarely seen in peripheral blood)	
Size	Large	Small	Small	Small
Shape	Very irregular	Compact often band forms	Compact	Compact
Vacuole	Prominent	Inconspicious	Inconspicious	Inconspicious
Chromatin	Dots or threads	Dots or threads	Dots or threads	Large irregular clumps
Pigment texture	Fine	Coarse	Coarse	Coarse
Color	Yellow brown	Dark brown	Black	Dark yellow brown
Quantity	Medium	Abundant	Medium	Medium
Distribution	Scattered fine particles	Scattered clumps and rods	Aggregated in two clumps	Scattered coarse particles

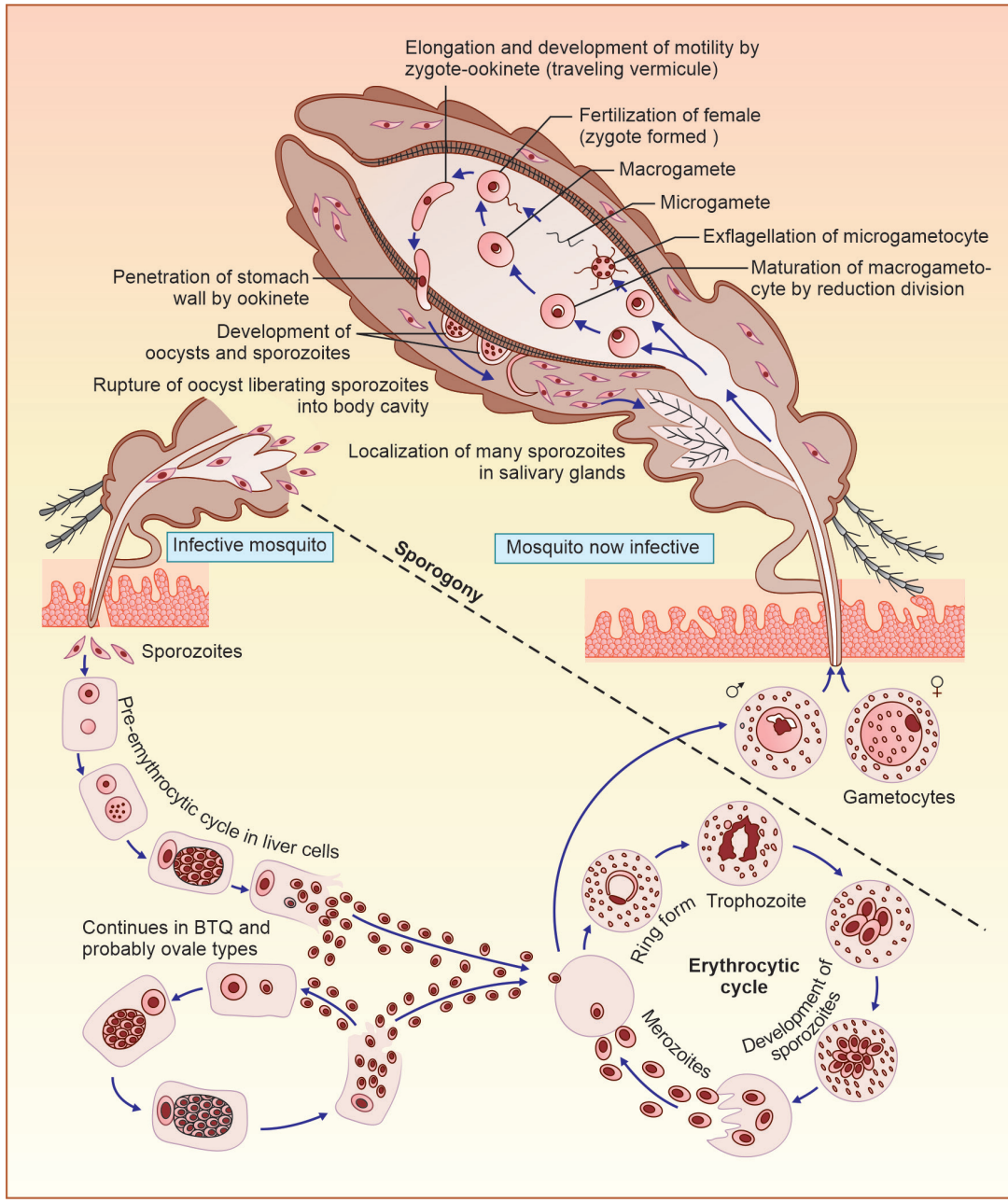
Morphology of Malarial Parasites (*Contd...*)

	P. vivax	*P. malariae*	*P. falciparum*	*P. ovale*
Immature schizonts			 (Rarely seen in peripheral blood)	
Size	Almost fills red cells	Almost fills red cells	Almost fills red cells	Almost fills red cells
Shape	Somewhat ameboid	Compact	Compact	Compact
Chromatin	Numerous irregular masses	Few irregular masses	Numerous irregular masses	Few irregular masses
Pigment	Scattered	Scattered	Scattered	Scattered
Immature schizonts			 (Rarely seen in peripheral blood)	
Size	Fills red cells	Nearly fills red cells	Nearly fills red cells	Fills of ¼ red cells
Shape	Segmented	Segmented daisy head	Segmented	Segmented
Merozoites				
14–24	6–12	6–32	6–12
Mean	16	8	24	8
Sixe	Medium	Large	Small	Large
Pigment	Aggregated in center (yellow brown)	Aggregated in center (dark brown)	Aggregated in center (black)	Aggregated in center (dark yellow brown)

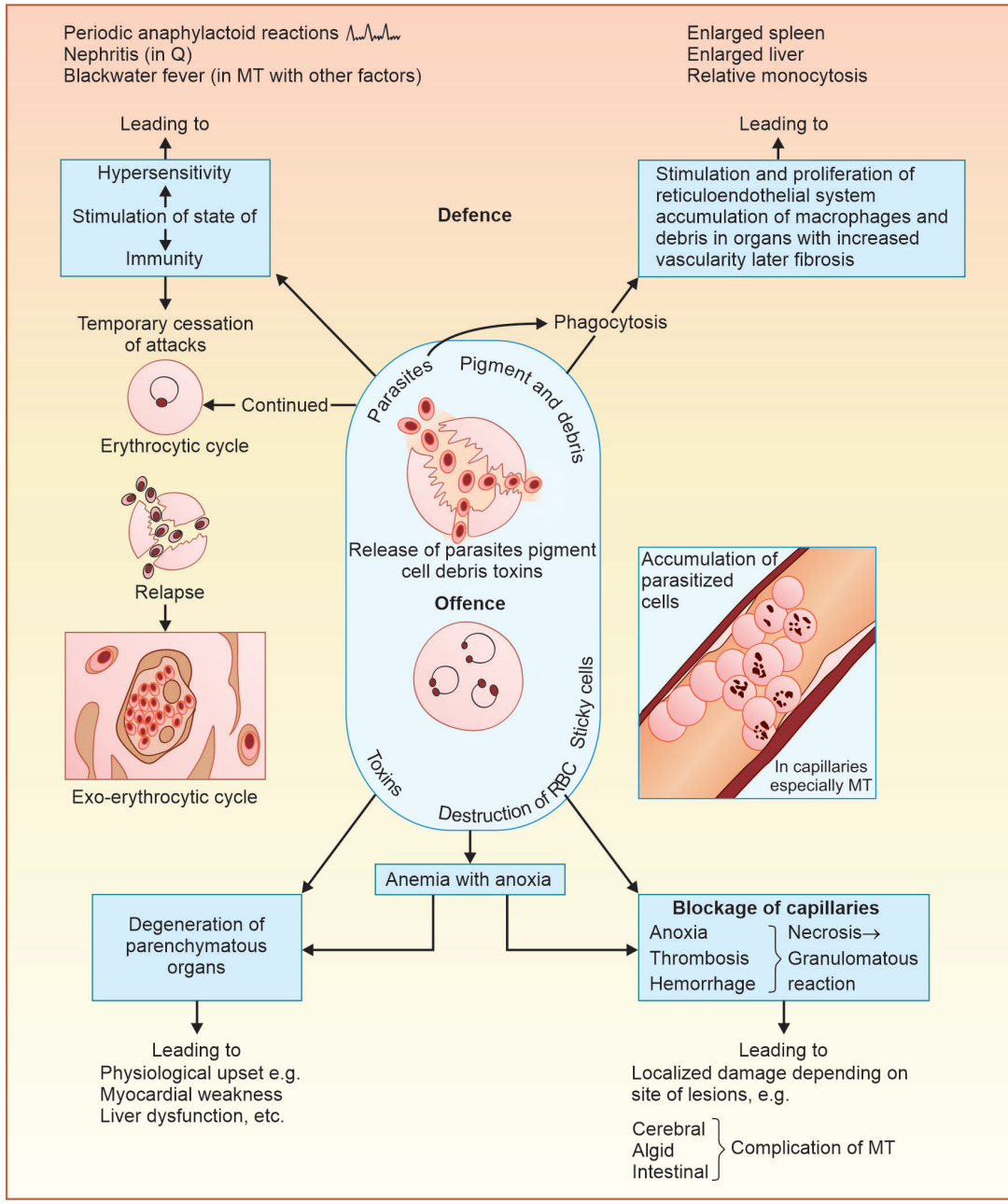
Morphology of Malarial Parasites (*Contd...*)

	P. vivax	*P. malariae*	*P. falciparum*	*P. ovale*
	Microgametocytes			
				
Time of appearance	3–5 days	7–14 days	7–12 days	12–14 days
Number in blood stream	Many	Scanty	Many	Scanty
Size	Fills enlarged red cell	Smaller than red cell	Larger than red cell	Size of red cell
Shape	Round or oval compact	Round compact	Kidney shaped bluntly round ends	Round compact
Cytoplasm	Pale blue	Pale blue	Reddish blue	Pale blue
Chromatin	Fibrils in skin with surrounding unstained area	As for *P. vivax*	Fine granules scattered throughout	As for *P. vivax*
Pigment	Abundant brown granules throughout	As for *P. vivax*	Dark granules throughout	As for *P. vivax*
	Microgametocytes			
				
Time appearance	3–5 days	7–14 days	7–14 days	12–14 days
Number in bloodstream	Many	Scanty	Many	Scanty
Size	Fills enlarged red cell	Smaller than red cell	Larger than red cell	Size of red cell
Shape	Round or oval compact	Round compact	Crescentic-sharply rounded or pointed ends	Round compact
Cytoplasm	Dark blue	Dark blue	Dark blue	Dark blue
Chromatin	Compact peripheral mass	As for *P. vivax*	Compact masses near center	As for *P. vivax*
Pigment	Small masses round periphery	As for *P. vivax*	Black granules round-nucleus	As for *P. vivax*

Life Cycle of Malarial Parasites

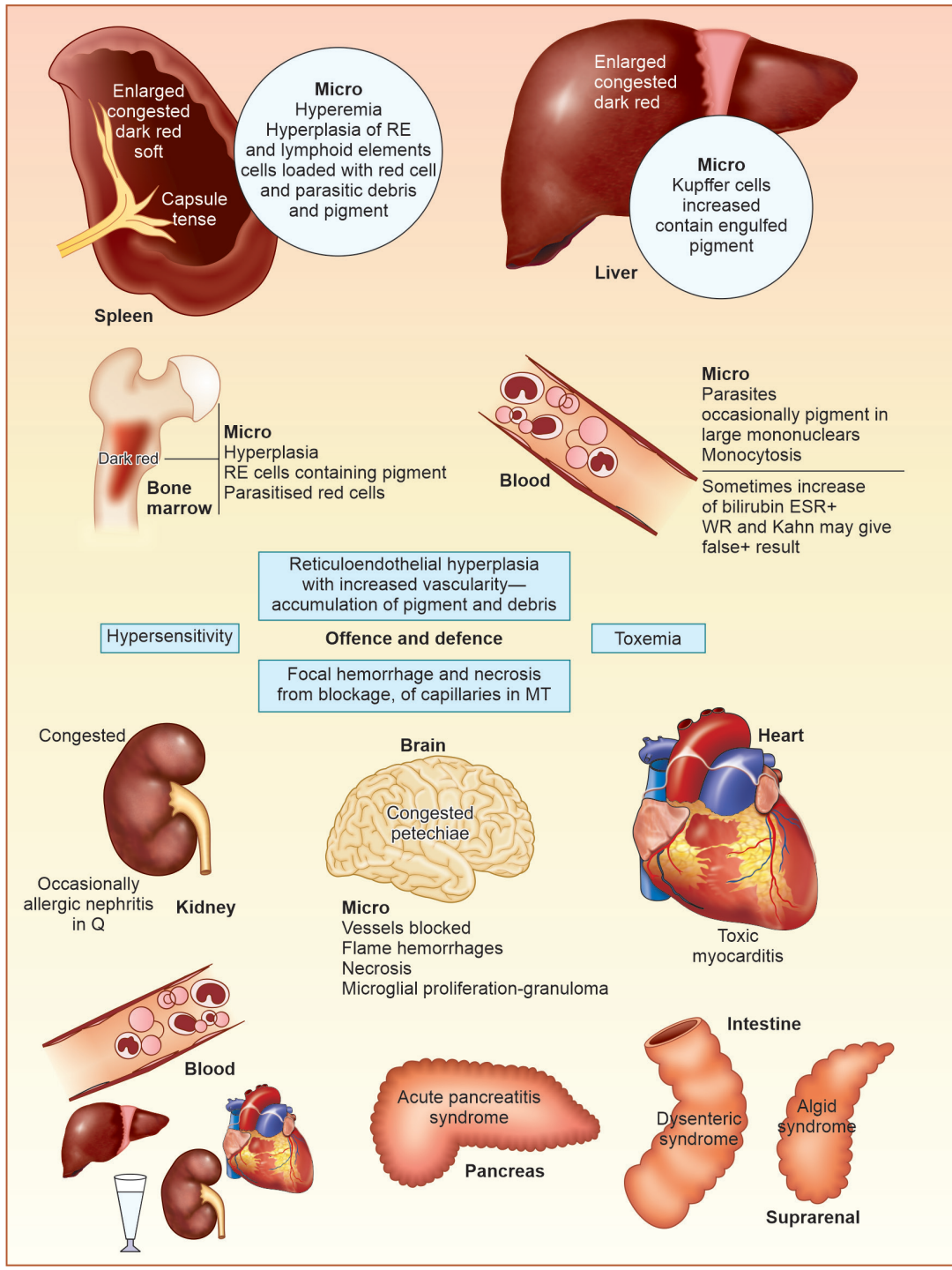


Pathogenesis of Malaria



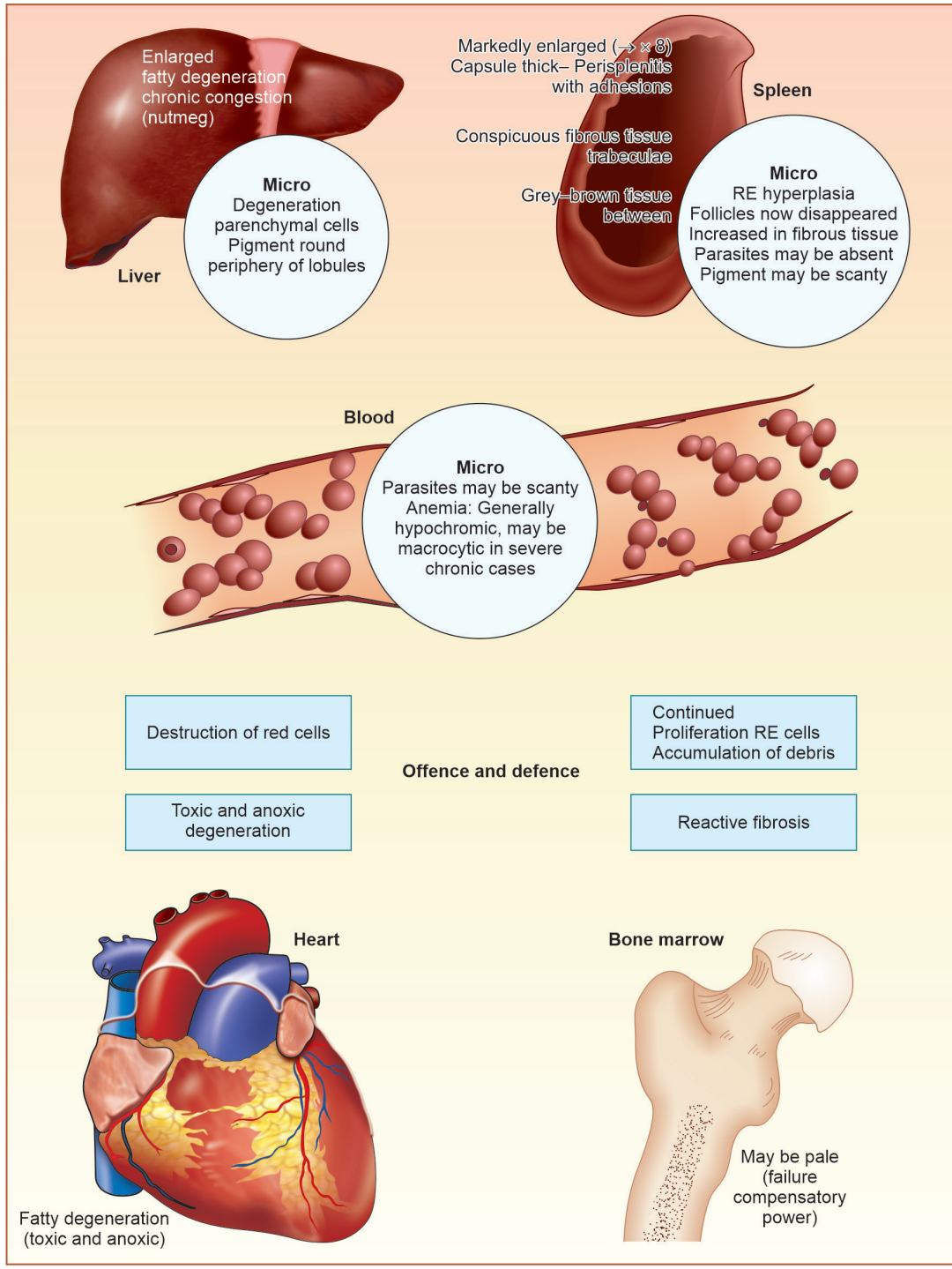
Pathology of Malaria

1. Acute Phase



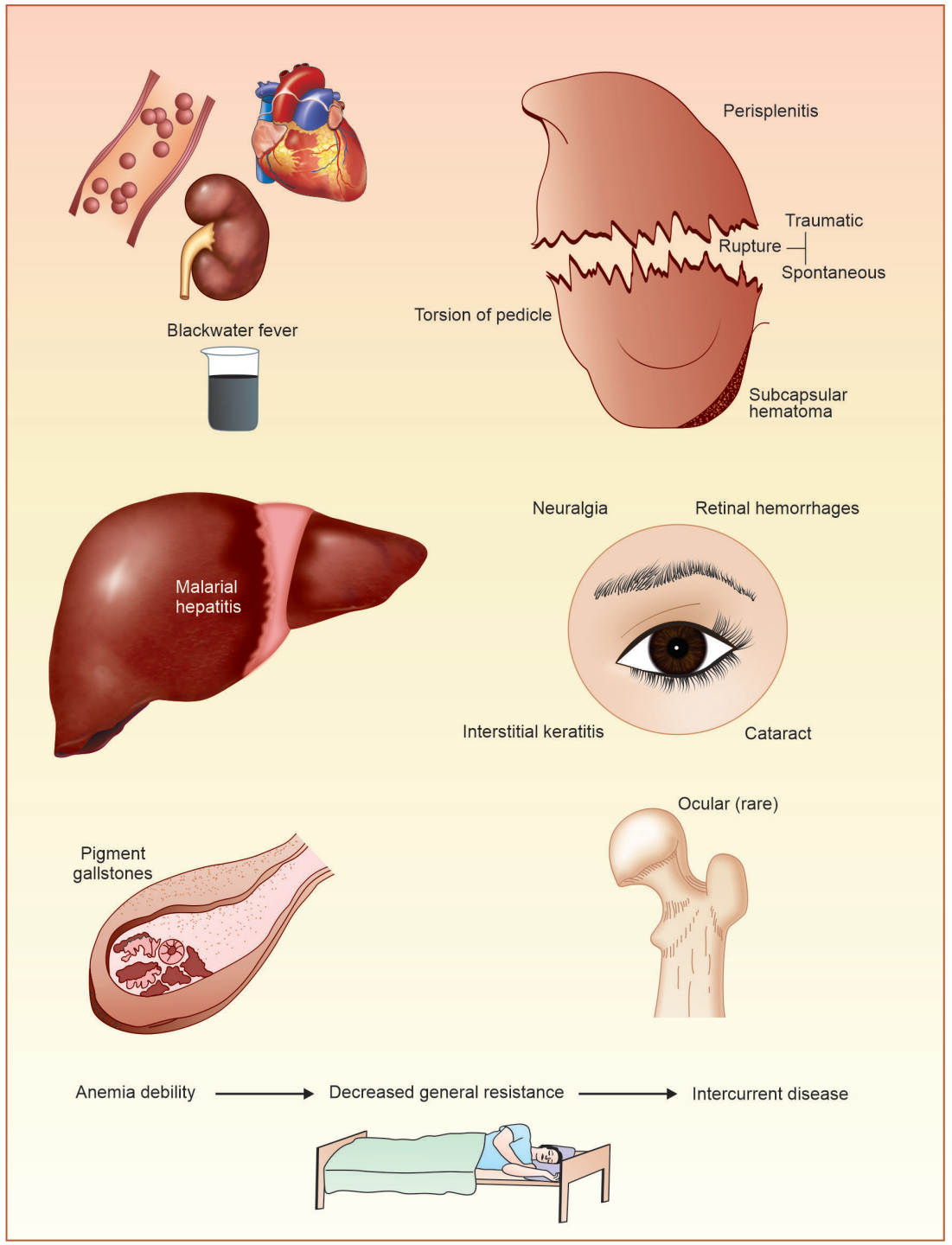
Pathology of Malaria

2. Chronic Phase



Pathology of Malaria

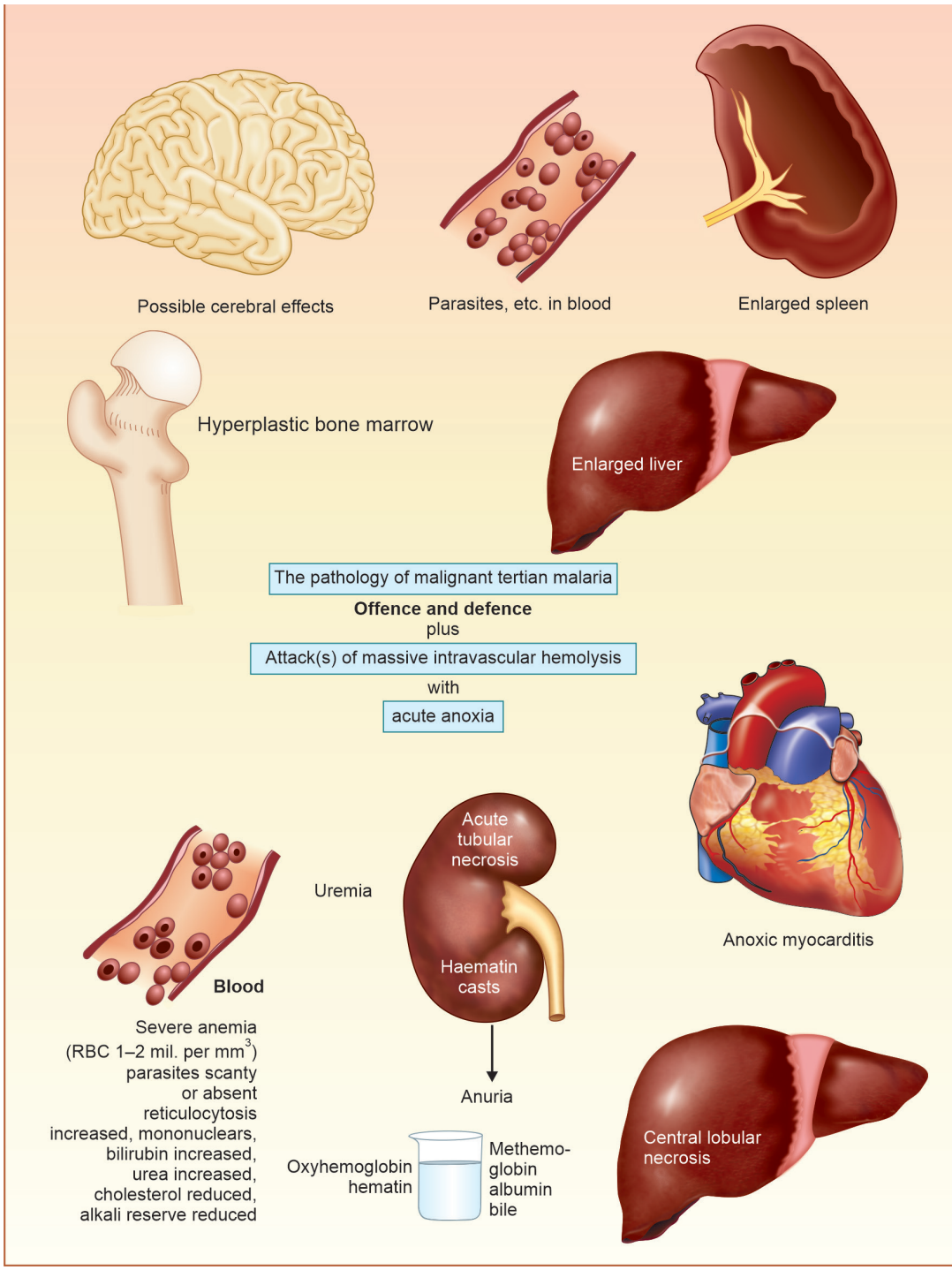
3. Complications and Sequelae



Pathology of Malaria

4. Blackwater Fever

Acute hemolytic attacks in MT malarias; associated with taking of quinine; numerous theories as to mechanism [d]



BLOOD FLAGELLATES OF MAN

Table 23.2: Blood flagellates of man

Prevalence depends upon presence of vertebrate reservoirs and proper insect hosts and varies with human habitation, habits, and agricultural practices.

Parasite and distribution	*Fly host*	*Enters man*	*Life cycle in man*	*Exit*	*Cycle in fly*	*Reservoir host*
Leishmania donovani Asia, Africa, tropical South America, Middle East, Mediterranean basin	Phlebotomus or Lutzomyia (Sandfly)	Plug of promastigotes (leptomonad) injected with bite of fly	Become amastigotes (leishmania or LD bodies) in RES macrophages	Sucked into fly with blood of host	Become promastigotes (leptomonads) in the intestine of fly	Man, dogs, foxes or other carnivores, wild rodents
Leishmania tropica Asia, South and Central America, Middle East, Europe	Phlebotomus or Lutzomyia (Sandfly)	-do-	Become amastigotes in endothelial cells of skin	-do-	-do-	Man, various wild rodents, possibly dogs
Leishmania brasiliensis Central and South America	Lutzomyia (Sandfly)	-do-	Become amastigotes in endothelial cells of skin and secondarily in mucous membranes of nasopharynx	-do-	-do-	Man, various wild rodents, possibly dogs
Trypansoma gambiense Central and West Africa	Glossina palpalis (Tsetse fly)	Fly bites; metacyclic trypomastigotes (trypanosomes) injected with saliva	Trypomastigotes in lymph and blood; later in spinal fluid	-do-	Become epimastigotes (crithidia) and then meta-cyclic trypomastigotes in the intestine and salivary glands of fly	Man, domestic animals
Trypanosoma rhodesiense Central and East Africa	Glossina morsitans (Tsetse fly)	-do-	-do-	-do-	-do-	Man, wild game animals (antelopes
Trypanosoma cruzi Central and South America	Panstrongylus megistus (kissing bug) and other reduvid bugs	Metacyclic trypomastigotes in feces scratched into skin or rubbed into mucous membrane of eye	Become amastigotes in tissue cell (particularly cardiac muscle) and trypomastigotes in bloodstream	Sucked into bug with blood of host	Become epimastigotes and then metacyclic trypomastigotes in the hind intestine of bug.	Man, dogs, cats, foxes, armadillo, opossum, rodents

*Varies in each area, in India and Africa, the dog apparently is not involved in *L. donovani* transmission. RES = Reticuloendothelial system.

Table 23.3: Blood flagellate diseases of man

Disease and etiology	*Clinical features*	*Laboratory diagnosis*
Kala-azar (visceral leishmaniasis) *L. donovani*	Chronic febrile disease, causing hyperplasia and then blockage of the reticuloendothelial system, particularly spleen and liver. Irregular fever (may spike twice a day) with chills, sweating, diarrhea, edema, cachexia, leukopenia, anemia. Splenomegaly and leukopenia charac- teristic. (Incubation period: 2–9 months) Untreated cases usually end fatally; with proper treatment, fatality is under 5%	Blood: Thick and thin smears, culture in NNN or Tobie's diphasic blood agar. CF test diagnostic; nonspecific tests such as Napier and Chopra serum tests for screening; skin tests for past infection. Nasal scrappings, lymph node biopsy, sternal marrow and splenic or hepatic aspirate. Stained smears, culture, inoculation of hamsters.
Oriental sore (cutaneous leishmaniasis) *L. tropica*	Endothelial cells and lymphoid tissue of skin parasitised. Itching red papule → scaling → crusted ulcer → ulcer enlargement → healing. May be multiple [Incubation period: several days to months, depending on strain, (1) dry (urban), relatively benign, slowly ulcerating form; or (2) moist (rural) acute, rapidly ulcerating zoonotic form.]	Ulcer curettings (from margin, not centre of ulcer): Stained smears, culture.
American leishmaniasis (espundia, forest yaws, uta; mucocutaneous leishmaniasis) *L. brasiliensis*	Initial ulcers similar to oriental sore, but this enlarges, producing weeping 'saucer' ulceration. Destructive and deforming secondary lesions occur at mucocutaneous junctions, particularly of nasopharynx. Produces fever, pain, malaise, and anemia (Incubation period: indeter- minate). Initial lesion—few days, complications—months to years. Nutrition probably very important in severity.	Ulcer curettings (from margin not centre, of smear): Stained smears, culture.
Sleeping sickness West African (*Trypanosoma gam-biense*) East African (*Trypanosoma rhodesiense*)	Local lesion at fly bite followed by fever, adenitis, rash, transitory edemas. May fulminate (*T. rhodesiense*) or go on to meningoencephalitis and meningomyelitis, with mental and physical wasting leading to coma and death (T. gambiense). (Incubation period: *T. gambiense,* 1–3 weeks; *T. rhodesiense,* 1–2 weeks.)	Blood: Thick and thin smears, concentration, culture. Spinal fluid sediment smears. Lymph node fluid: Smears and culture

Contd.

Table 23.3: Blood flagellate diseases of man		
Disease and etiology	*Clinical features*	*Laboratory diagnosis*
Chagas' disease *T. cruzi*	Acute, usually in children: Febrile illness with generalized adenopathy lasting a few months; placental infection common. Chronic cardiac involement, particularly of right ventricle, consisting of degeneration of cardiac muscle. Patient seldom lives beyond age 50. Megacolon or megaesophagus a sequel. Anemia. Romana's sign (palpebral edema) most probably an allergy to insect bite (Incubation period: 1-2 weeks)	Blood: Thick and thin smears only in initial phases. Culture. Xenodiagnosis. Complement fixation most reliable serologic test

* Direct blood film stain positive except in heavy infections; buffy coat smear or culture better.*

Basis of Serum Biochemical Tests for Leishmaniasis

These tests assess alterations in serum proteins particularly serum gamma globulin. The positive results are obtained after 2–3 months or more.

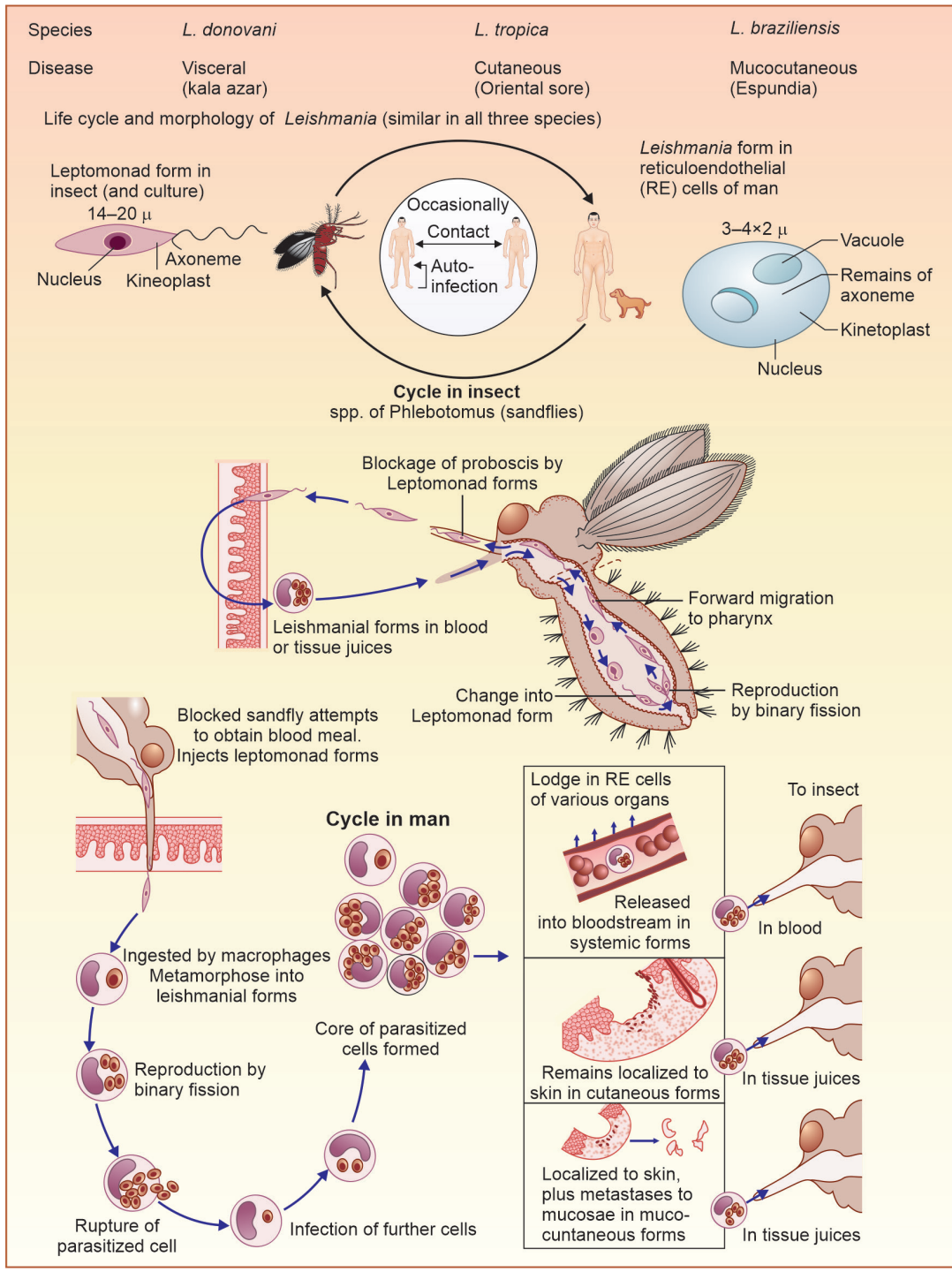
Napier's aldehyde test: When serum of the patient is treated with formaldehyde, it causes flocculation and opacity. To 1 mL of patient's serum, add 2 drops of formaldehyde—if flocculation and opacity occur within 10 minutes, the test is strongly positive. If this change occurs in about 2 hours time, it is labeled as weakly or doubtful positive.

Chopra's antimony test: Dilute patient's serum 10 times with normal saline. To 1 mL of diluted serum, add 2 drops of 4% urea stibamine solution. Immediate appearance of flocculum and turbidity indicates a positive test.

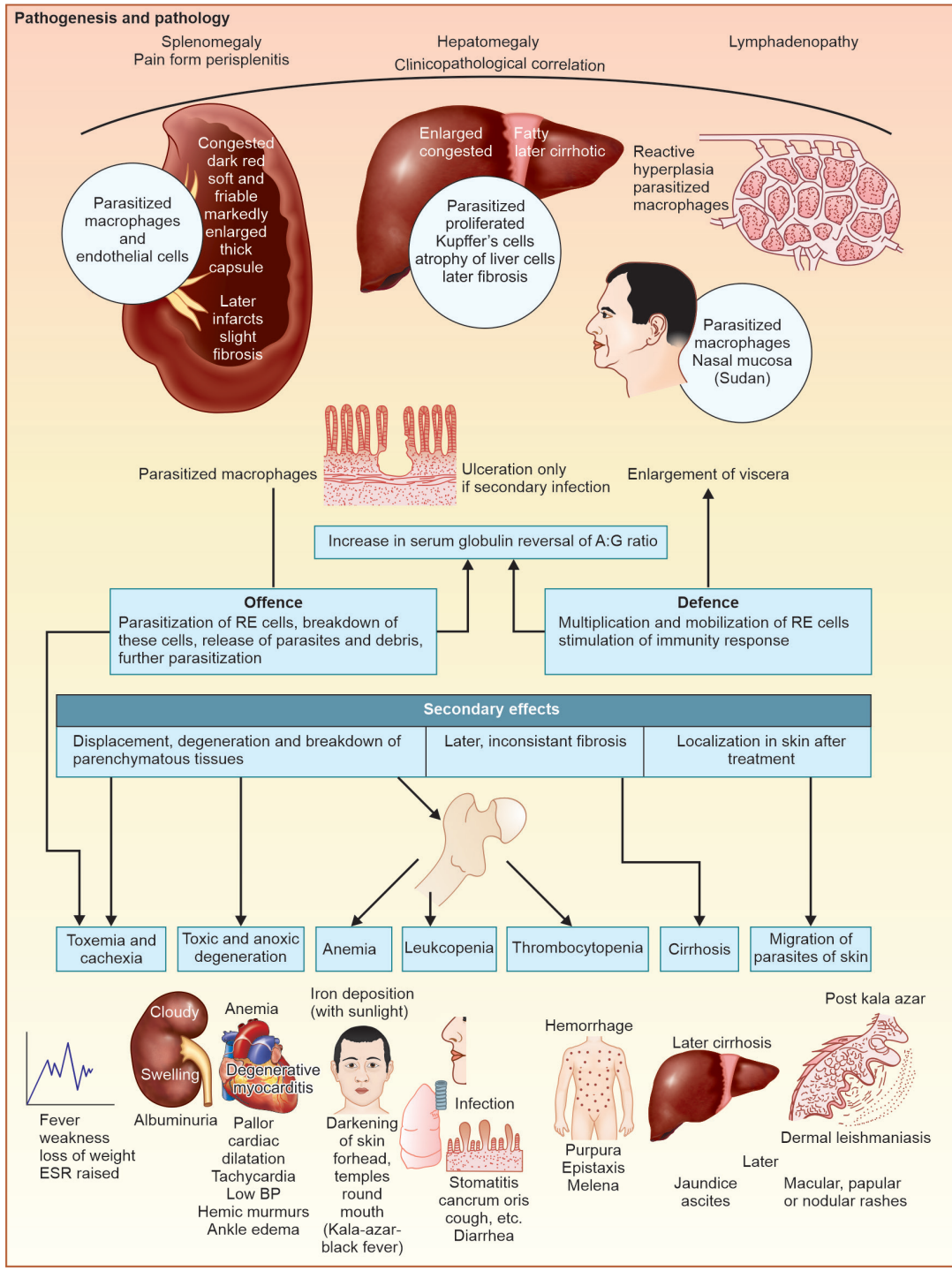
For exact determination of individual classes of gamma globulins, immunoturbidometric or nephalometric techniques may be used. However, for screening purposes and normal routine clinical testing, the given above tests are quite satisfactory.

ELISA techniques are ideal.

LEISHMANIASIS



VISCERAL LEISHMANIASIS (KALA AZAR) CAUSED BY *LEISHMANIA DONAVANI*



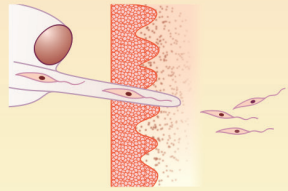
CUTANEOUS LEISHMANIASIS (ORIENTAL SORE, CHICLERO'S DISEASE, UTA, ETC.) CAUSED BY *LEISHMANIA TROPICA*

Blocked sandfly injects Leptomonad forms | Core of cells parastized by LD bodies formed | Acanthosis cellular infiltration | Pressure necrosis and ulceration | Secondary injection | Granulation | Healing (2–12 months) with depressed pigment scar

Remains localized in skin

Ulcer with sharp edges and surrounding induration

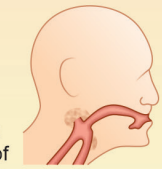
Mucocutaneous leishmaniasis (Esupundia) caused by *Leishmania braziliensis*



Blocked sandfly injects Leptomonad forms

Cutaneous manifestations like oriental sore but often weeping ulcers

Spread to mucosae of mouth-nose, larynx, pharynx, eat



Paralized cell inflammatory infiltration nacrosis Later reactive fibrosis

Secondary effects in loose mucosal tissues

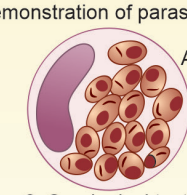
Edema and capillary involvement interferance with local blood supply Necrosis—Extensive destruction	Secondary infection Deep erosion locality Spread of infection to lungs or elsewhere	Healing with fibrosis

Leading to

Extensive disfiguring lesion | General constitutional upset (fever, pain, anemia) | Bronchopneumonia and septicemia

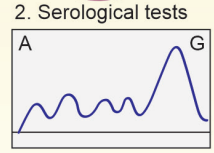
Diagnosis of leishmaniasis

1. Demonstration of parasite by stained film
   Culture (NNN)
   Animal inoculation (Hamsters)



In Blood
Bone marrow
Lymph node juice
Nasal scraping (Sudan)
Spleen puncture } Not free from danger
Liver puncture }

2. Serological tests



Specific
Nonspecific

CFT (antigen, leishmania cultures)
Depending on reversal AG ratio
Formal gel (Napier)
Aldehyde (Chopra)
Euglobulin precipitation (Sia)
Electrophoresis

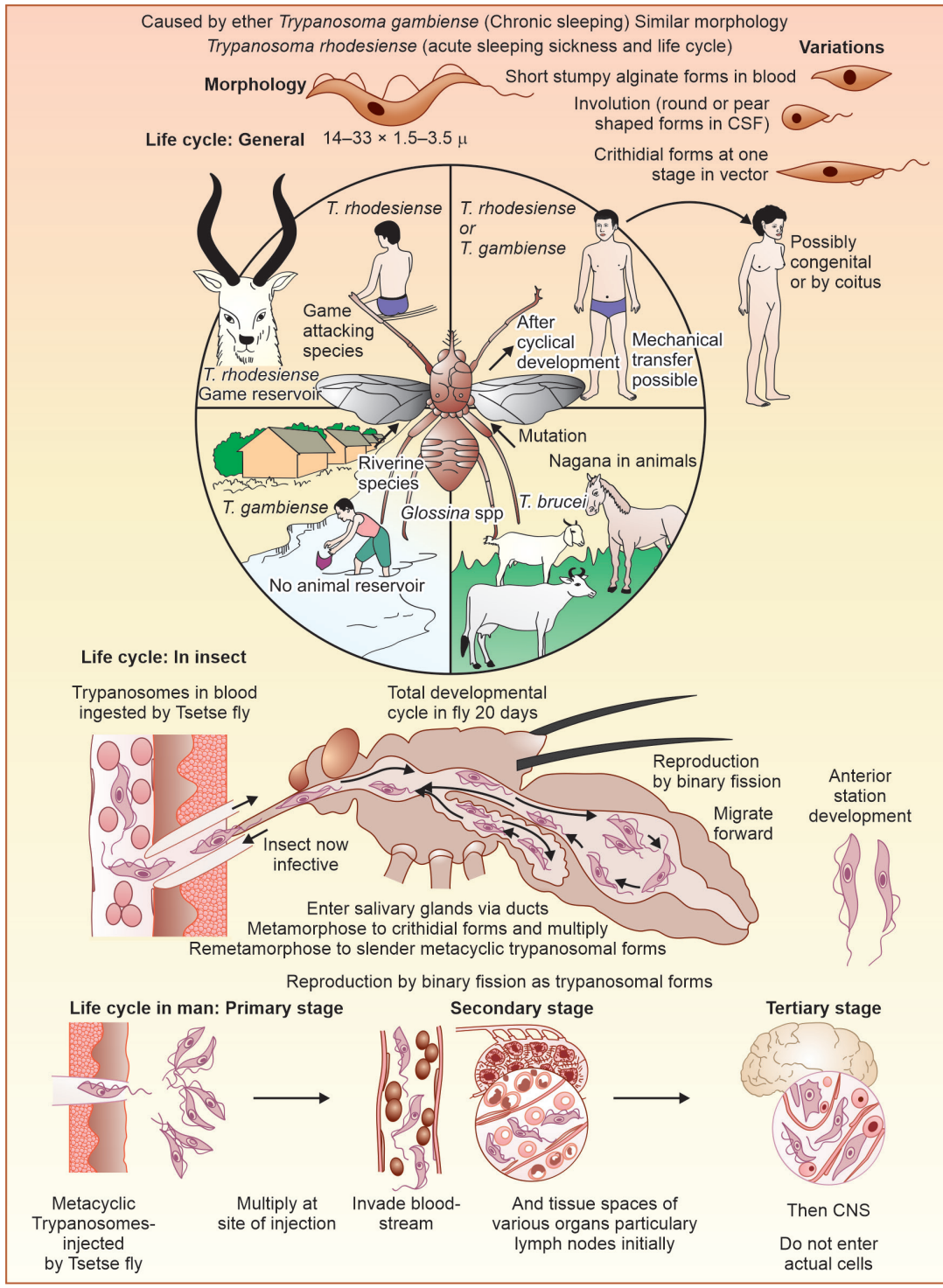
Cutaneous and mucocutaneous

1. Demonstration of parasite by stained film
   Culture
   Animal inoculation

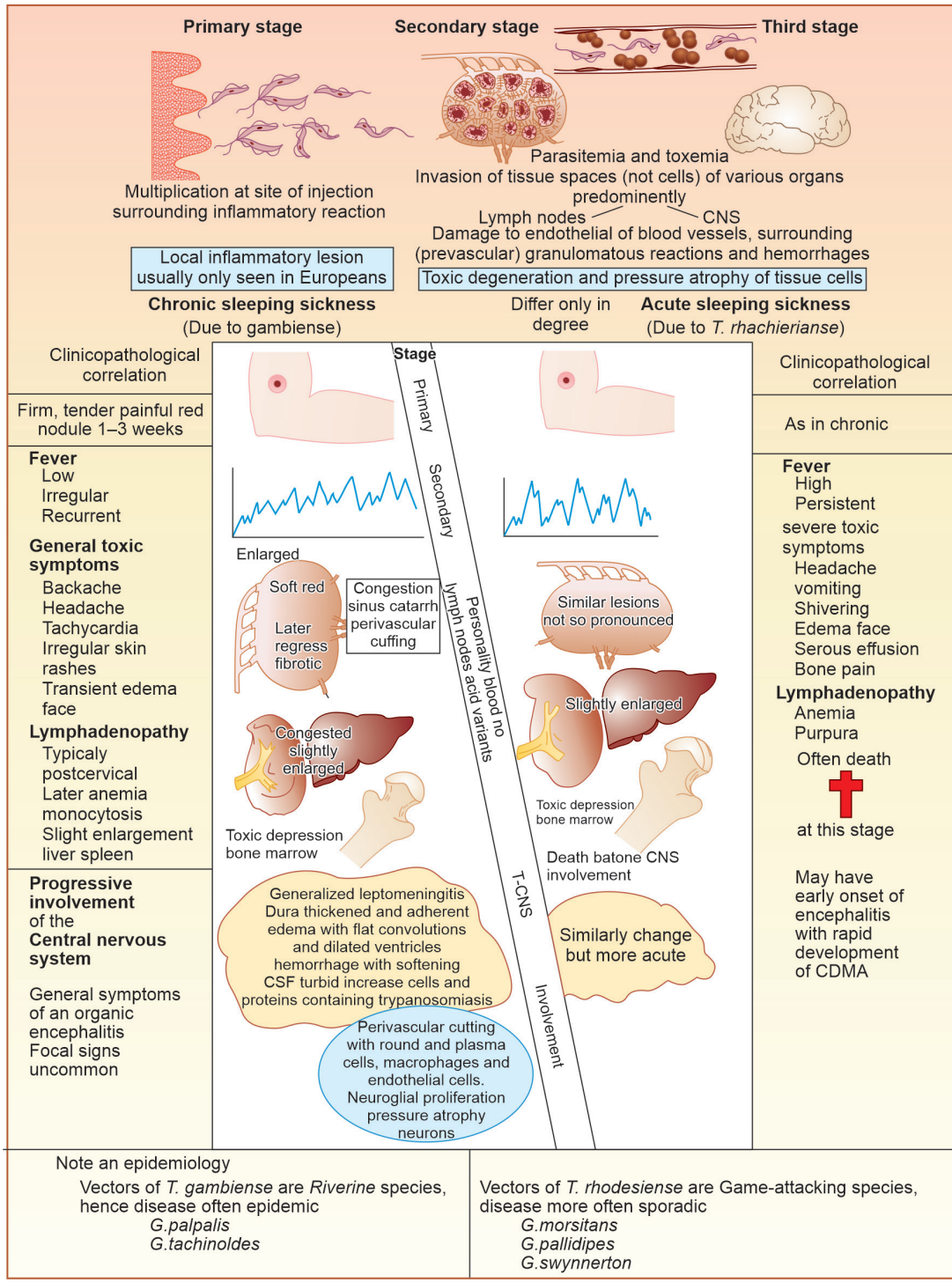
In Serum from indurated edge of ulcer
Biopsy of margin ulcer
Mucosal scraping in mucocutaneous type

2. Intradermal test (Montenegro). Antigen culture of appropriate *Leishmania*
3. Serological tests in mucocutaneous type

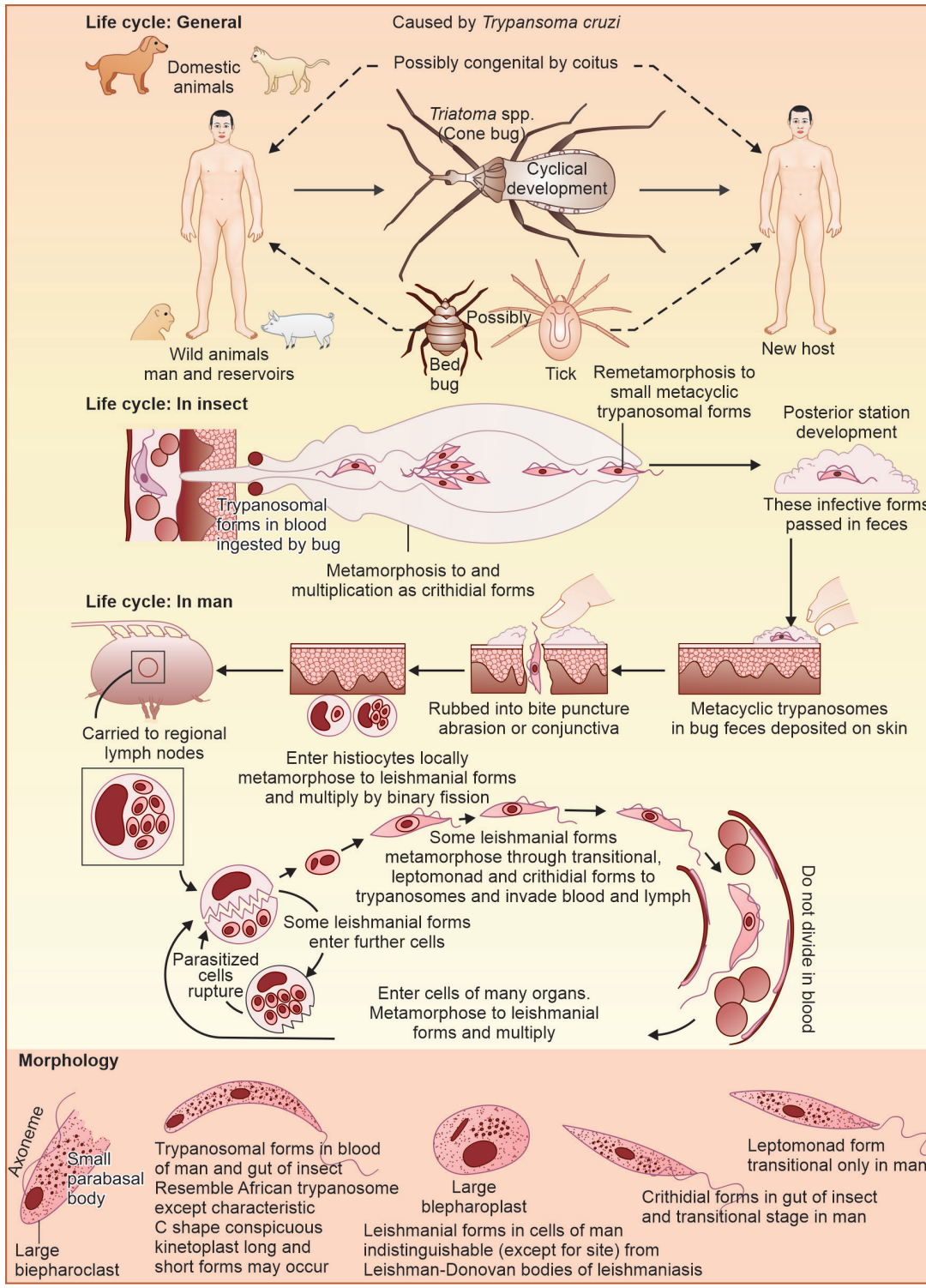
TRYPANOSOMIASIS: AFRICAN TYPE: SLEEPING SICKNESS


Caused by ether Trypanosoma gambiense (Chronic sleeping) Similar morphology
Trypanosoma rhodesiense (acute sleeping sickness and life cycle)
Variations
Morphology
Short stumpy alginate forms in blood
Involution (round or pear shaped forms in CSF)
Life cycle: General
14–33 × 1.5–3.5 μ
Crithidial forms at one stage in vector
T. rhodesiense
T. rhodesiense or T. gambiense
Possibly congenital or by coitus
Game attacking species
After cyclical development
Mechanical transfer possible
T. rhodesiense Game reservoir
Mutation
Nagana in animals
Riverine species
T. gambiense
Glossina spp
T. brucei
No animal reservoir
Life cycle: In insect
Trypanosomes in blood ingested by Tsetse fly
Total developmental cycle in fly 20 days
Reproduction by binary fission
Migrate forward
Anterior station development
Insect now infective
Enter salivary glands via ducts
Metamorphose to crithidial forms and multiply
Remetamorphose to slender metacyclic trypanosomal forms
Reproduction by binary fission as trypanosomal forms
Life cycle in man: Primary stage
Secondary stage
Tertiary stage
Metacyclic Trypanosomes-injected by Tsetse fly
Multiply at site of injection
Invade blood-stream
And tissue spaces of various organs particulary lymph nodes initially
Then CNS
Do not enter actual cells

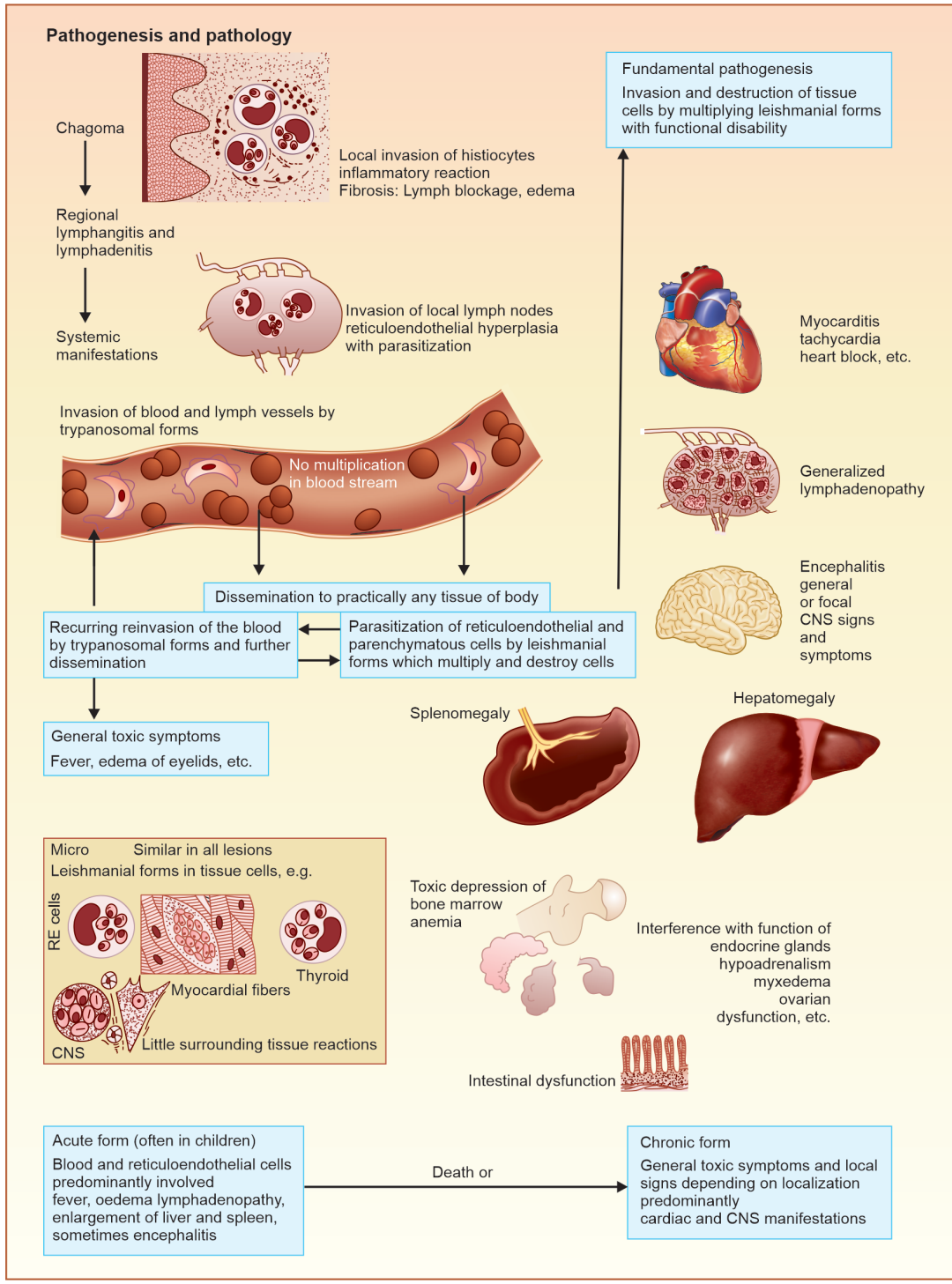
AFRICAN TRYPANOSOMIASIS (SLEEPING SICKNESS) (CONTD...)



TRYPANOSOMIASIS SOUTH AMERICAN TYPE: CHAGAS' DISEASE


Life cycle: General
Caused by Trypansoma cruzi
Domestic animals
Possibly congenital by coitus
Triatoma spp. (Cone bug)
Cyclical development
Wild animals man and reservoirs
Possibly
Bed bug
Tick
New host
Remetamorphosis to small metacyclic trypanosomal forms
Life cycle: In insect
Posterior station development
Trypanosomal forms in blood ingested by bug
These infective forms passed in feces
Metamorphosis to and multiplication as crithidial forms
Life cycle: In man
Carried to regional lymph nodes
Rubbed into bite puncture abrasion or conjunctiva
Metacyclic trypanosomes in bug feces deposited on skin
Enter histiocytes locally metamorphose to leishmanial forms and multiply by binary fission
Some leishmanial forms metamorphose through transitional, leptomonad and crithidial forms to trypanosomes and invade blood and lymph
Some leishmanial forms enter further cells
Parasitized cells rupture
Enter cells of many organs. Metamorphose to leishmanial forms and multiply
Do not divide in blood
Morphology
Axoneme
Small parabasal body
Large biepharoclast
Trypanosomal forms in blood of man and gut of insect Resemble African trypanosome except characteristic C shape conspicuous kinetoplast long and short forms may occur
Large blepharoplast
Leishmanial forms in cells of man indistinguishable (except for site) from Leishman-Donovan bodies of leishmaniasis
Crithidial forms in gut of insect and transitional stage in man
Leptomonad form transitional only in man

SOUTH AMERICAN TRYPANOSOMIASIS (CHAGAS' DISEASE) (CONTD...)


Pathogenesis and pathology
Chagoma
Regional lymphangitis and lymphadenitis
Systemic manifestations
Local invasion of histiocytes
inflammatory reaction
Fibrosis: Lymph blockage, edema
Invasion of local lymph nodes
reticuloendothelial hyperplasia
with parasitization
Fundamental pathogenesis
Invasion and destruction of tissue cells by multiplying leishmanial forms with functional disability
Myocarditis
tachycardia
heart block, etc.
Generalized lymphadenopathy
Encephalitis general or focal CNS signs and symptoms
Invasion of blood and lymph vessels by trypanosomal forms
No multiplication in blood stream
Dissemination to practically any tissue of body
Recurring reinvasion of the blood by trypanosomal forms and further dissemination
Parasitization of reticuloendothelial and parenchymatous cells by leishmanial forms which multiply and destroy cells
General toxic symptoms
Fever, edema of eyelids, etc.
Splenomegaly
Hepatomegaly
Micro Similar in all lesions
Leishmanial forms in tissue cells, e.g.
RE cells
Thyroid
Myocardial fibers
CNS
Little surrounding tissue reactions
Toxic depression of bone marrow anemia
Interference with function of endocrine glands
hypoadrenalism
myxedema
ovarian dysfunction, etc.
Intestinal dysfunction
Acute form (often in children)
Blood and reticuloendothelial cells predominantly involved
fever, oedema lymphadenopathy, enlargement of liver and spleen, sometimes encephalitis
Death or
Chronic form
General toxic symptoms and local signs depending on localization predominantly
cardiac and CNS manifestations

TISSUE ROUNDWORMS OF MAN, CHIEFLY FILARIAE

Table 23.4: Tissue roundworms of man—chiefly filariae, life cycle

Parasite and distribution	*Intermediate host*	*Mode of human infection*	*Site of worm maturation*	*Larvae found in*	*Exit*	*Larval development in*	*Reservoir host*
Wuchereria bancrofti ***Brugia malayi*** Tropics and subtropics	Culex, Aedes, Anopheles, Mansonia, and other mosquitoes	Filariform larvae actively leave mosquito at time of biting, usually enter skin via puncture	In lymph vessels and nodes	Bloodstream (nocturnal periodicity)* certain strains sub- or non-periodic	Microfilariae taken up by insect when biting	Thoracic muscles; larvae migrate to proboscis (8–15 days)	For *B. malayi:* Cats, dogs monkeys
Loa loa Tropical Africa	Chrysops (deer or mangrove flies)	Probably same	In subcutaneous tissues	Bloodstream (diurnal periodicity)	Same	Abdomen, larvae migrate to proboscis	Primates?
Onchocerca volvulus Tropical Africa Venezuela, and mountainous portions of central Africa	Simulium (buffalo gnat)	Probably same	Same, host reaction, produces nodule around cluster of adults	Subcutaneous tissue; eye (no periodicity)	Same	Thoracic muscles; larvae do not concentrate in proboscis	Man
Dracunculus medinensis Africa, India, Far and Middle East, Indonesia	Cyclops (water flea)	Infected cyclops accidentally ingested in water	Digested out of cyclops, than worm migrates into tissues; mature female passes to skin (may be 1 m long)	Adult female in skin causes host to form blister near head of worm. Blister then filled with larvae, bursts when skin immersed in water, discharging larvae. New blister later forms as female moves to new site			Man

*Periodicity of microfilarial appearance in peripheral blood. May be nocturnal in circulating blood from 8 pm, after which microfilariae confined to pulmonary capillaries

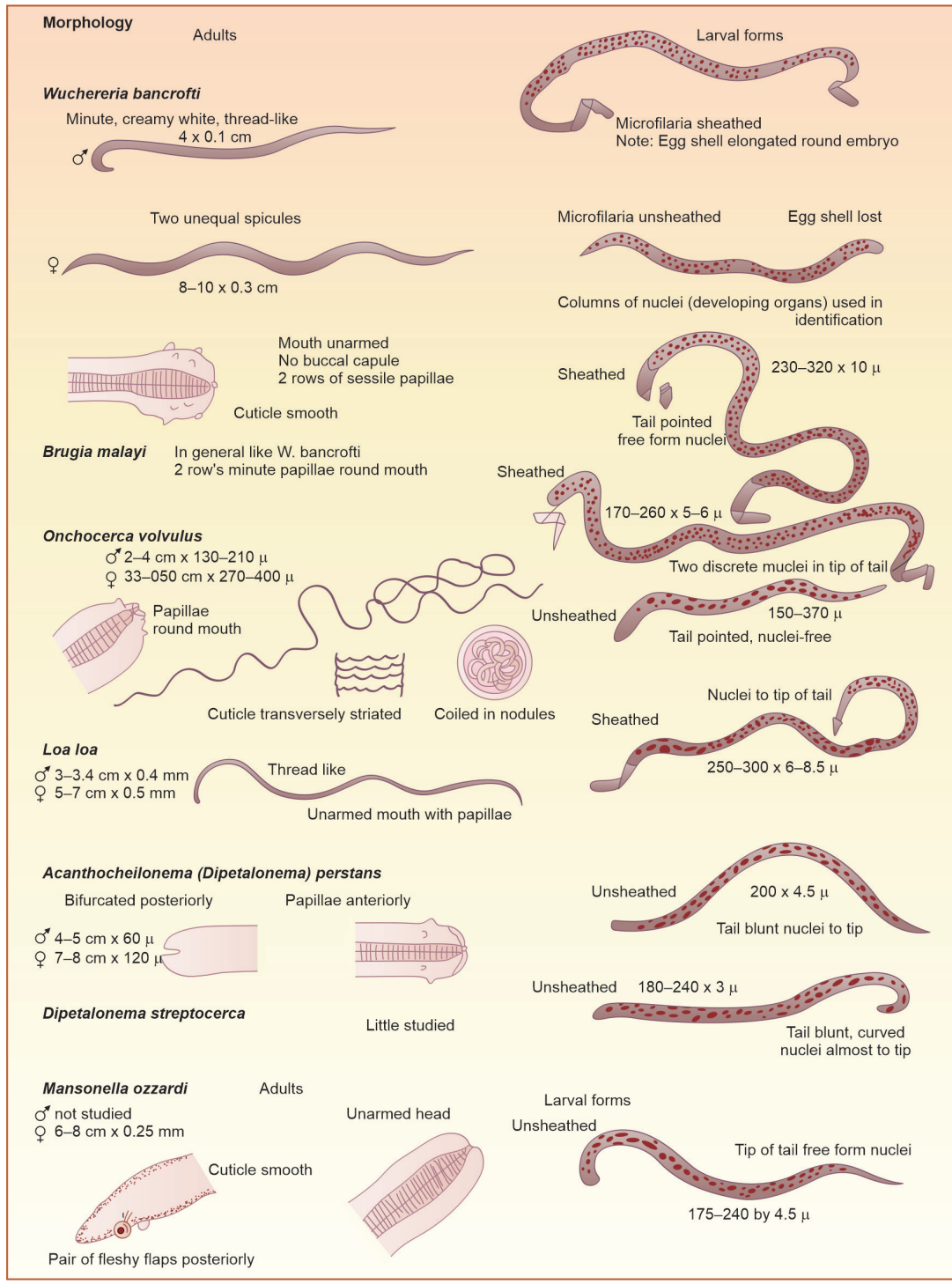
Table 23.5: Tissue roundworms of man—chiefly filariae, pathology

Disease and etiology	*Clinical features*	*Laboratory diagnosis*
Bancroft's filariasis *W. bancrofti* **Malayan filariasis** *B. malayi*	Filariae in afferent lymph nodes (of lower extremities, male genitalia, vulva, mammary gland) may cause inflammation followed by intensely fibrotic reaction involving whole area in a mass of scar tissue. Pain, fever, chills, toxemia, eosinophilia. Chronic stage (after disappearance of micro- filariae) varies from microscopic lesion to lymph varicosity to marked elep- hantiasis. High eosinophilia and superficial lymphadenopathy in Malayan filariasis. Pathology probably a general and focal sensitization response.	Blood: Thick and thin smears, concentration. Intradermal test gives useful group filaria reaction, indirect hemagglutination, fluorescent antibody tests.
Loiasis *Loa loa*	Fugitive swelling ("Colabar swelling") in skin due to local edema and skin reaction against migrating adult worm. The latter commonly moves across surface of eyeball or under skin at bridge of nose (best times for removal with local anesthesia). Eosinophilia with occasional proteinuria produced.	Microfilariae in nodule aspirate or skin snip; repeated skin snips may be required; scapular cutaneous region area of choice, manifestations: eosinophilia ocular lesions, pruritus may follow diethylcarbamazine (Hetrazan) therapy
Onchocerciasis *O. volvulus*	Small cutaneous fibrous skin nodules with filariae entwined in centre. Microfilariae in nodule, in neighboring tissue, or in normal skin far from nodules, rarely in blood. Skin reactions particulary common in Africa; reduction of elastic fibers, depigmentation, progressive thickening, pruritus, papulovesi- cular lesions or hyperkeratotic patches with microfilariae in scrapings. Eosinophilia and transient urticaria during incubation. Ocular involvement leading to blindness, a common result of prolonged infection. Conjunctiva and vitreous humor with numerous microfilariae. Pathology due to mechanical action, toxins, hypersensitive response of patient; ocular symptoms after 7-9 years.	
Dracontiasis *D. medinensis*	Asymptomatic until reddish papular lesion appears, usually on legs. Lesion forms blister bearing head of female worm and numerous larvae. Blister bursts when immersed in water, releasing larvae. Urticaria, pruritus, allergic symptoms, eosinophilia. Accidental rupture of worm may produce intense inflammatory reaction with secondary infection.	Local lesion with head of worm and larvae in blister. X-ray reveals calcified worm; reflected light shows worm under skin. Intradermal test

Eosinophilic lung describes a host allergic response to migration of microfilariae that are trapped in lungs, producing an allergic, asthma-like response. Probably, caused by human filariae or closely related species, as completion of worm development and microfilariae production required. Diagnosed by clinical signs, eosinophilia (3000 or more absolute count, usually over 35%), high hemagglutination or complement fixation titre that drops following Heterazan therapy, elevated ESR, mottled lung lesions visible under X-ray.

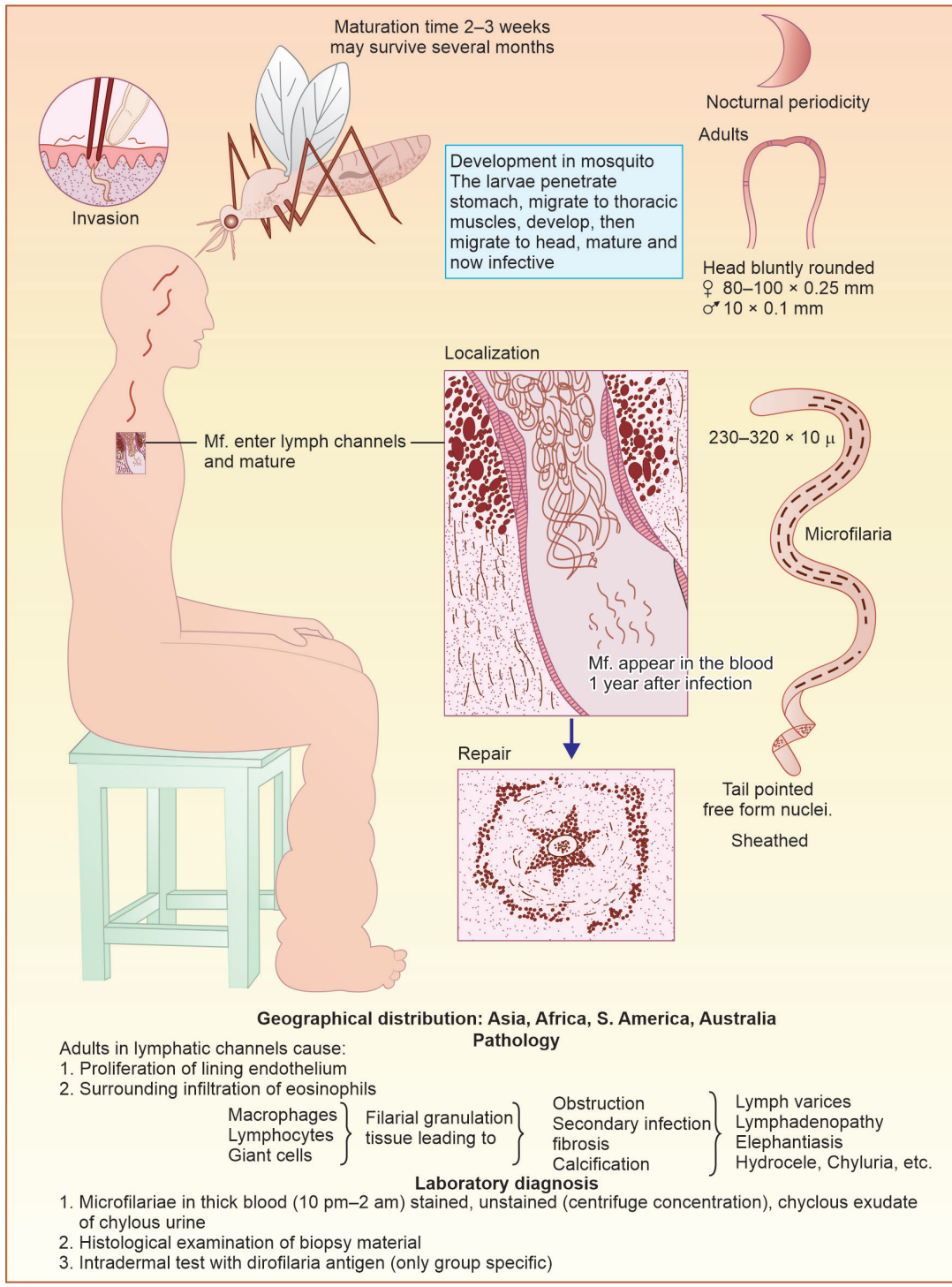
PLASMID NEMATODES

Filarial Worms



PLASMID NEMATODES (*CONTD...*)

Wuchereria bancrofti



Geographical distribution: Asia, Africa, S. America, Australia

Pathology

Adults in lymphatic channels cause:

1. Proliferation of lining endothelium
2. Surrounding infiltration of eosinophils

Macrophages, Lymphocytes, Giant cells → Filarial granulation tissue leading to → Obstruction, Secondary infection, fibrosis, Calcification → Lymph varices, Lymphadenopathy, Elephantiasis, Hydrocele, Chyluria, etc.

Laboratory diagnosis

1. Microfilariae in thick blood (10 pm–2 am) stained, unstained (centrifuge concentration), chyclous exudate of chylous urine
2. Histological examination of biopsy material
3. Intradermal test with dirofilaria antigen (only group specific)

Plasmid Nematodes (Filarial Worms) (*Contd...*)

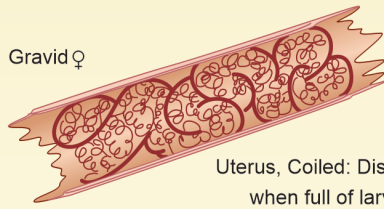
Described in various situations, life cycle obscure

D. conjuntivae Lives encysted in subcutaneous tissue
D. immitis A dog parasite. lives in cardiac chamber
Usual source of antigen (group specific) for intradermal and complement fixation tests for filarial infections in man

Dracunculus medinesis
(The guinea worm)

Morphology ♀ Very long, filiform 70–120 cm × 0.9–7 mm

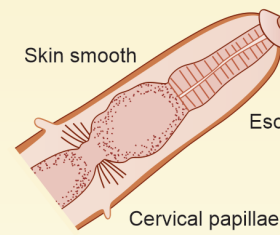
♂ Little studied in man 12–29 × 0.4 mm

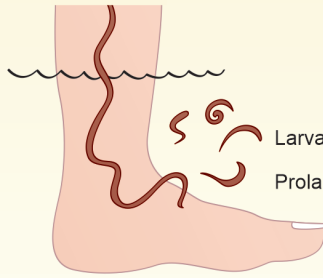


Uterus, Coiled: Distended when full of larvae

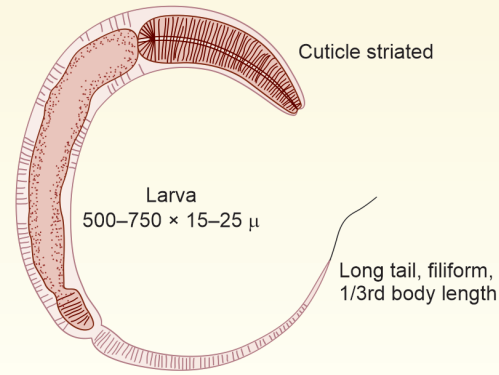
Larviposition
Some 3 million per worm

Anterior end

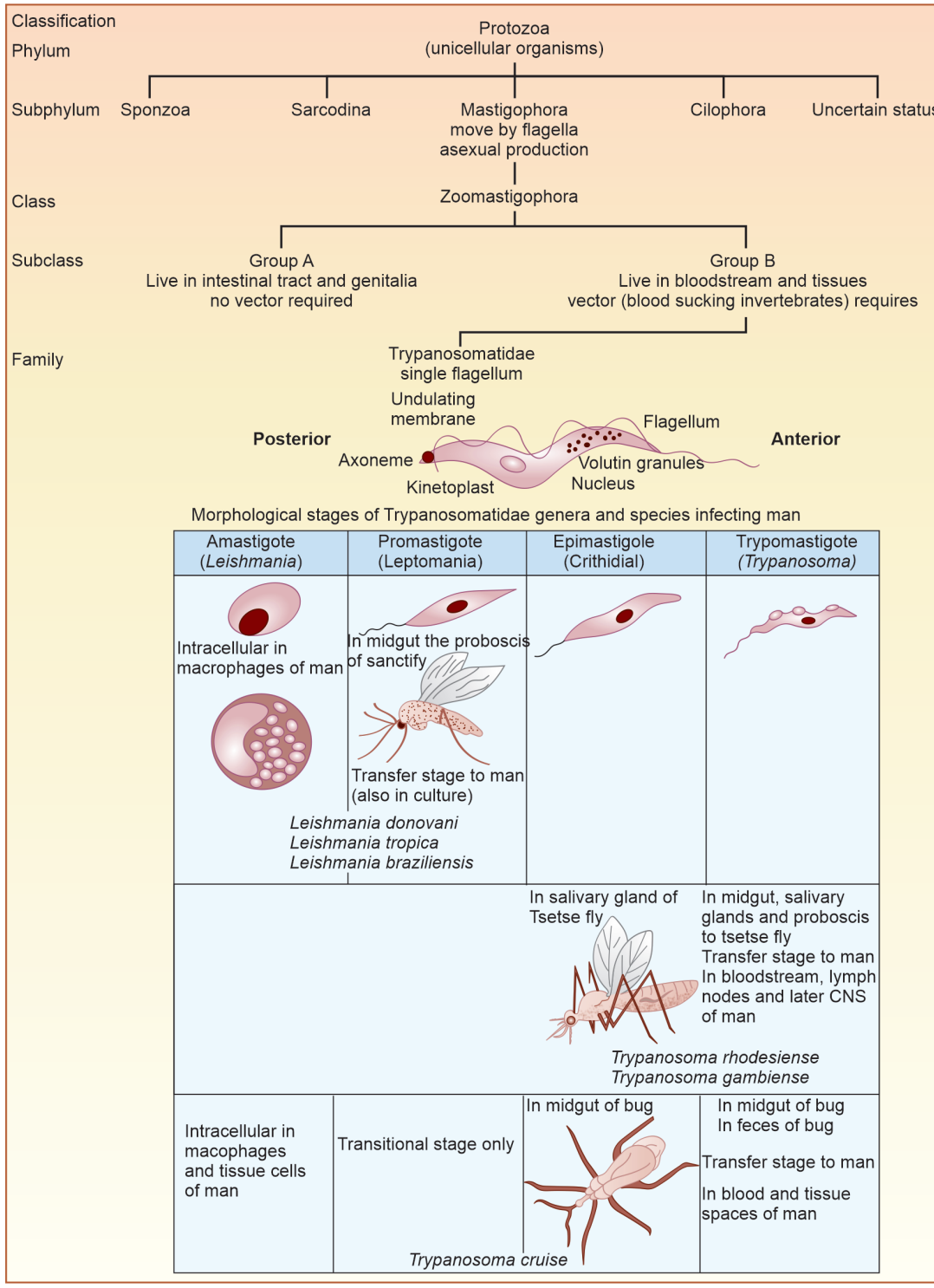




In subcutaneous tissue



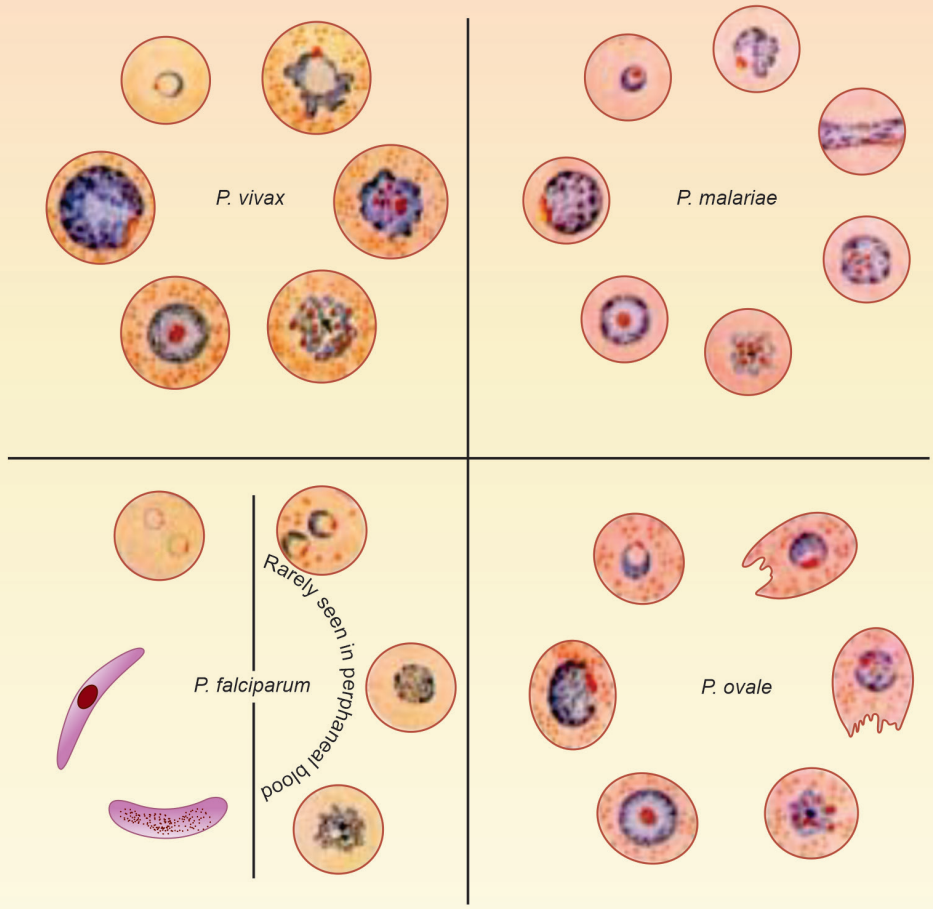
Body Fluid and Tissue Flagellates (Causing Leishmaniasis and Trypanosomiasis)



Laboratory Diagnosis of Malaria

Laboratory diagnosis of Malaria

Malarial parasites in thin film of blood. Stained by Leishman and Giemsa



Thick film of blood stained by field or Giemsa

In thick or thin film of bone marrow

Concentration
Serological
Culture
} **Techniques academic rather than practical**

Black water fever

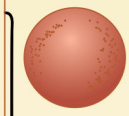
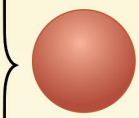
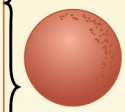
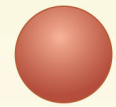
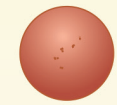
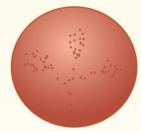
In addition to above

Spectroscopic examination of urine and feces

Tests to exclude other causes of hemoglobinuria

(e.g., Donath–Landsteiner reaction for cold agglutinins)

Malaria Species Identification in the Mosquito—Pigment in Oocysts

Oocysts	*P. vivax*	*P. malariae*	*P. falciparum*	*P. ovale*
Length of cycle in days	9	15–21	10	15
Size in μ	10–46	5–44	8–60	9–37
pigment				
Color	Greenish brown	Dark brown or nearly black	Blackish	Dark brown or nearly black
Texture	Fine	medium coarse	Very coarse	Medium coarse
Number of grains	50–100	30	10–20	50–60
Pattern				
Days				
3	None			
4	Typical prince of wales feather design	Distributed some clumping		
5			 Concentrated at periphery in double row often	Concentrated at periphery in semicircles or dotted lines
6				
7	Obscured by nuclei			
8		 Increased clumping	 Mainly obscured	Most clearly defined, dotted line often croses
9				
10				
After 10		 If visible, clumped at periphery	Seldom visible	 Mainly obscured
Recapitulation of distinctive features	 Prince of wales feather design	Distributed then clumped	Peripheral in rows	Semicircular or crossed line design

Recapitulation

Recapituation

The essential difference between:

	African trypanosomiasis (sleeping sickness)	South american trypanosomiasis (Chagas' disease)	Visceral leishmaniasis (kala azar)
Caused by	*Trypanosoma gambiense or Trypanosoma rhodesiense*	*Trypanosoma cruzi*	*Leishmania donovani*
Vector	Glossina app. (Tsetse flies)	Trialomida app. (Cone-nosed bugs)	Phlebotomus spp. (Sand files)
Cycle in vector	Anterior station development	Posterior station development	Anterior station development
Stage ingested then	Trypanosomal	Trypanosomal	Leishmanial
	Multiply and move forward Enter salivary glands via duct	Metamorphose to crithidia	Metamorphose to Leptomonad multiply
	Metamorphose to crithidia multiply	Multiply move backward	Move backward block proboscis
	Metamorphose to metaphysis	Metamorphose to metacyclic trypansomes	
	Trypanosomes		
	Infective: Injected in saliva	Infective: Passed in bug feces	Infective: Injected form proboscis
Cycle in man			
Form injected	Metacyclic trypanosoma	Metacyclic trypanosoma	Leptomonad
	None-remain as trypanosomes	Enter histiocytes locally and become leishmanial	Enter histiocytes location and become leishmanial
Multiplication (at by binary fission)	As trypanosomes in blood and tissue spaces	As leishmanial forms in cells	As leishmanial forms in macrophage cells
Then	Remain in bloodstream as trypanosomes	Carried to regional nodes: some leishmanial forms infect further histiocytes: Some metamorphose through transitional leptomonad and crithidial forms to gain blood as trypanosomes. Do not multiply as such but disseminated, enter further tissue cells, metamorphase to leishmanial forms and multiply	Further dissemination as leishmanial form only
Pathogenesis	Circulating, multiplying trypanosomes cause parastema and toxemia damaging tissues	Parasitization and destruction of all type of tissue cells circulating trypanosomes produce toxemia	Parasitization and destruction RE cell
Pathological effects	Mainly general toxemia lymphadenopathy CNS involvement	General toxemia local functional disability of whichever tissues invaded, especially lymph nodes, heart CNS	General toxemia from breakdown of RE cells Proliferation of RE cells (mainly spleen liver, bone marrow, lymph nodes) NO CNS involvement
Clinico-pathological correlation	Acute *(T. rhodesiense)* fever and severe toxemia Chronic (*T. gmbiense*) Fever Lymphadenopathy Encephalitis	Fever enlarged nodes, spleen and liver Protein manifestation depending on localization in tissues especially Cardiac CNS syndromes	Fever Anemia enlarged liver and spleen

TOXOPLASMOSIS (Fig. 23.1)

Toxoplasmosis a common infection, is caused by *Toxoplasma gondii*. Most human infections are acquired from cats. Affected patients may be symptomless or suffer a brief illness with lymphadenopathy and fatigue. Severe infection, most commonly seen in foetus (congenital toxoplasmosis) or in patients with immunodeficiency, is associated with extensive damage to the brain, eyes, muscle, heart and lungs. Diagnosis is usually confirmed by positive serology. Lymph node biopsy may be needed. Rarely, the trophozoite forms are present in blood monocytes.

BABESIOSIS (Fig. 23.2)

Babesiosis is a tick-borne disease caused by protozoan parasites of the genus *Babesia*. The disease affects a number of animal species and is occasionally transmitted to humans. In most patients the illness is mild and characterized by fever, malaise, myalgia, mild hepatosplenomegaly and hemolytic anemia. Occasional patients who have had previous splenectomy are known to have developed a more fulminant infection with massive intravascular haemolysis which may prove to be fatal.

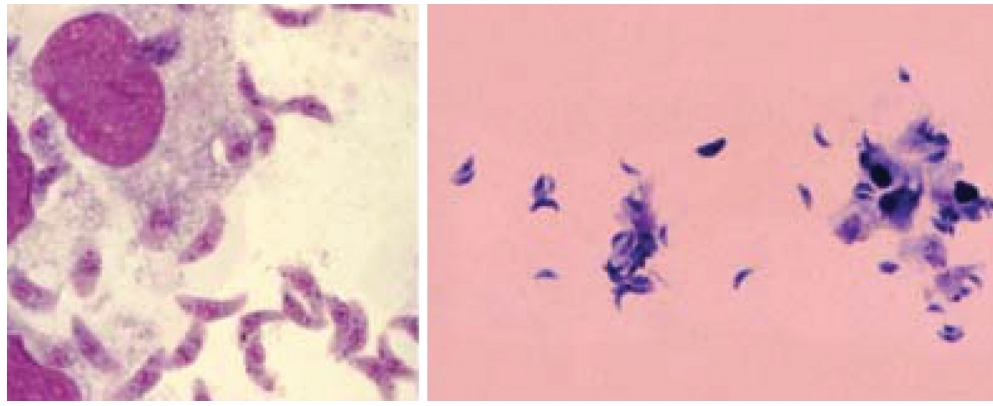


Fig. 23.1: *Toxoplasma gondii* trophozoites

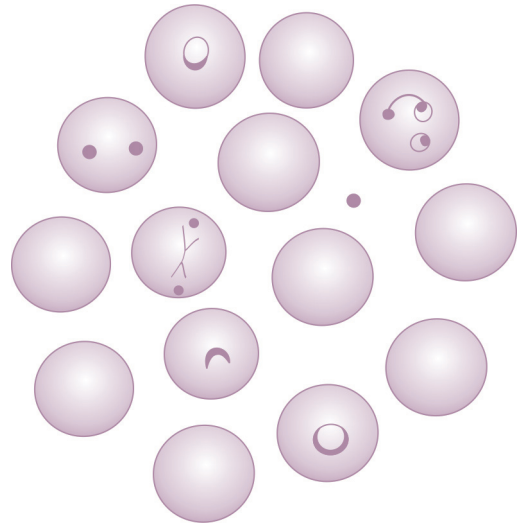


Fig. 23.2: RBCs infected with *Babesia* species

The majority of human infections have been caused by *Babesia microti,* a species usually infecting rodents. Several cases In splenectomised patients have been due to *Babesia* bovis, a species, associated with redwater fever in cattle. Diagnosis is made by finding the trophozoites, which resemble small ring forms of *Plasmodium falciparum* in RBCS.

BARTONELLOSIS (Fig. 23.3)

Bartonella bacilliformis causes a severe febrile hemolytic anemia. The disease occurs in inhabitants of the Andes mountains in Peru, Columbia and Ecuador. The characteristic rod-shaped coccobacilli are found in RBCs. The infection is known as Oroya fever and Carrion's disease and is transmitted by phlebotomus sandflies.

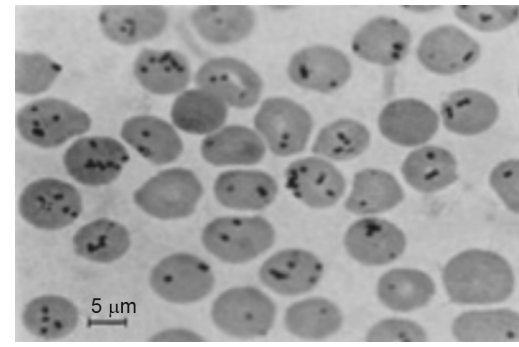


Fig. 23.3: *Bartonella* species infecting the RBCs

RELAPSING FEVER (FIG. 23.4)

Various spirochaetes of the genus *Borrelia* cause relapsing fever. Louse-borne relapsing fever is a human disease caused only by *Borrelia recurrentis,* but tick-borne relapsing fever is a zoonosis caused by a number of different species. It is during the febrile period of the disease that the organisms are present in the blood with the production of antibodies, the *Borrelia* disappear and the patient becomes afebrile. Relapses occur, after 7–10 days, with the production of new antigenic variants of the organisms. In louse-borne relapsing fever there is usually a single relapse, while in the tick- borne forms of the disease multiple relapses may occur.

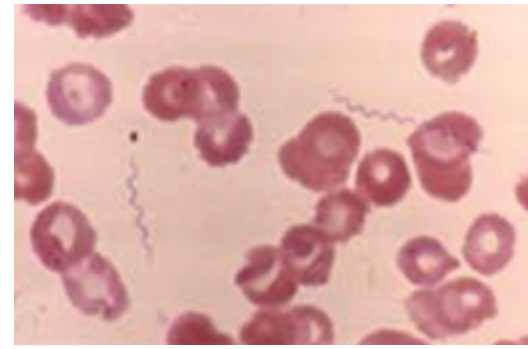


Fig. 23.4: *Borrelia* organisms as seen in the peripheral smear

CHAPTER

24

Practical Hematology

The blood consists of a fluid of complicated and variable composition, the plasma, in which are suspended erythrocytes (red blood cells—RBCs), leukocytes (white blood cells—WBCs) and platelets. By using an anticoagulant, the formed elements can be separated from plasma. When blood coagulates, the fluid that remains after separation of the clot is called serum. Serum = Plasma – fibrinogen. The techniques of hematology are concerned mainly with the cellular formed elements of blood, their number or concentration, the relative distribution of various types of cells and the structural or biochemical abnormalities that promote disease.

WAYS OF OBTAINING BLOOD

CAPILLARY OR PERIPHERAL BLOOD

For hematologic exercises, venous blood obtained from a vein is better. However, for total and differential blood counts and for hemoglobin estimation, blood can be taken by pricking: (i) the lobe of the ear, (ii) the palmar surfaces of the tip of the finger, (iii) in infants, from the plantar surface of the (a) heel, or (b) the great toe. The puncture should be about 3 mm deep. An edematous or a congested part should not be used. If the area to be punctured is cold and cyanotic, warm it by massaging or else erroneous results may be obtained. Clean the site with spirit or alcohol, let dry and puncture. Wipe off the first drop of blood, never press out blood. Having obtained the requisite amount of blood, let the patient apply slight pressure over the area with sterile swab (Fig. 24.1).

VENOUS BLOOD (VENIPUNCTURE)

Reassure the patient about what is to be done. Inspect the veins, use a tourniquet if needed (Fig. 24.2). Use a syringe of a size according to the amount of blood required. Needles

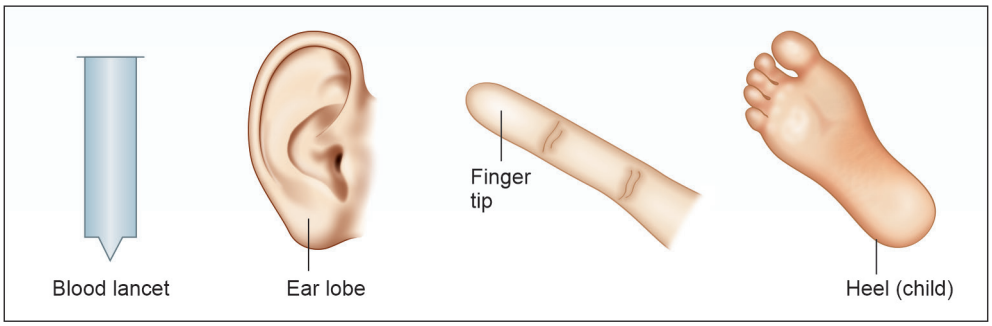


Fig. 24.1: Sites for obtaining blood skin puncture

of gauge less than 22 should be used and be 1–1½ inches long. Instead of a tourniquet, one can use a sphygmomanometer cuff, apply pressure that is midway between systolic and diastolic pressure. Ask the patient to open and close his fist several times. Take aseptic precautions, and puncture the vein. Sometimes, it may be difficult to obtain blood at the first instance, in such a case, first the skin around the vein is punctured and then the vein is punctured, the moment needle enters the vein, blood flows back into the syringe. If still blood has not been obtained withdraw the needle and in many instances blood would be obtained. Having withdrawn blood, loosen the tourniquet and ask the patient to open his fist. Let the patient apply a sterile gauze piece with gentle pressure over the area. The patient should apply pressure gently with three fingers. Why three fingers? Simply because the site of skin puncture is not the same as the site of venipuncture. Make sure that the bleeding has stopped before the patient leaves. In infants blood may be secured from femoral or external jugular vein. Wash the syringe after dispensing the blood in the right container. Transfer blood from syringe into the container gently (not through the needle). Ideally use disposable syringes and needles.

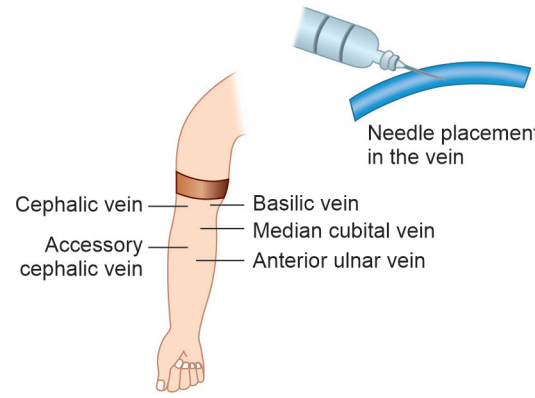


Fig. 24.2: Sites for obtaining blood by venipuncture from forearm

Complications

Immediate

Hematoma and syncope. Hematomas can be avoided by applying adequate gentle pressure at the site and for syncope a physician should be brought to take care. In any case, let the patient lie down if he or she is already not and raise the foot end. Some patients with a bleeding tendency may overbleed.

Late Local Complications

Thrombosis of the vein. Rarely thrombophlebitis may occur. Apply Thrombophob® if it happens. Seek professional medical help.

Late-general Complications

Transmission of serum hepatitis, AIDS by using contaminated needle and syringe.

Clinical Alert

1. If oozing from the puncture site is difficult to stop, elevate area and apply a pressure dressing. Stay with the patient until bleeding stops.
2. Never draw blood for any laboratory test from the same extremity that is being used for IV fluids, IV medications or blood transfusion.
3. In patients with leukemia or agranulocytosis and in others with lowered resistance, the finger-prick and ear lobe puncture are more likely to cause infection than venipuncture. If capillary sample is necessary in these patients, the cleansing agent should remain in contact with the skin for 7–10 minutes. Alcohol is not bactericidal, povidone iodine (Betadine®) is the cleansing agent of choice on leukemic patients.

ANTICOAGULANTS

On letting blood stand, it clots after some time. Anticoagulants are agents that prevent clotting when mixed with blood in an appropriate proportion. The common pathway of the clotting mechanism is represented as under:

$$\text{Prothrombin} \xrightarrow[Ca^{++}]{\text{Thromboplastin}} \text{Thrombin}$$

$$\text{Fibrin} \xleftarrow{\text{Thrombin}} \text{Fibrinogen}$$

(insoluble) Thrombin + Ca++ (soluble)

Fibrin with blood cells with entrapped constituents comprise a clot.

The thromboplastin released by damaged tissue, or platelets converts inactive prothrombin into active thrombin in the presence of calcium ions. Thrombin converts soluble fibrinogen into insoluble fibrin clot in the presence of calcium ions. Anticoagulants commonly used for hematological investigations are as follows.

EDTA

Ethylenediamine tetraacetic acid (EDTA), disodium or potassium salt. EDTA acts by chelating calcium and preserves cellular element better than oxalates. Eight milligrams of the salt is enough for anticoagulating 3–4 mL of blood.

- EDTA can be used for hemograms, ESR (Wintrobe's method), platelet count, DLC and peripheral smear examination.

Advantages of EDTA

- Cellular morphology is preserved better, even 2–3 hours after blood collection
- As platelet clumping is prevented, EDTA is a better anticoagulant for platelet counts
- EDTA 2K salt is recommended for CBC, is more water soluble (1.5 + 0.25 mg/mL of blood).

Disadvantages of EDTA

- When in excess, EDTA shrinks RBCs and leukocytes. If in excess of 2 mg/mL:
  - PCV is significantly reduced
  - MCHC is proportionately increased
  - Platelets swell and disintegrate, therefore, a fallaciously high platelet count may be obtained
- It cannot be used for coagulometry applications

Making EDTA Bulbs

Four grams of disodium or dipotassium salt is added to 100 mL of deionized water. About 0.2 mL of this solution is added to chemically clean vials, the vials are later kept in an incubator or hot air oven till complete liquid dries up (may take 1–2 hours at 60–80°C). White layer of anticoagulant can be seen at the bottom. This bulb is for 3–4 mL of blood, as it contains 8 mg of EDTA per vial.

OXALATES

These act by chelating calcium, calcium oxalate is formed as insoluble precipitate. These are used for blood chemistry and hematocrit.

- *Potassium oxalate* (2–3 mg/mL of blood) but it may cause shrinkage of cells. Not used anymore.

- *Double oxalate* used for ESR and hematocrit. Potassium oxalate and ammonium oxalate are used together in a ratio of 2:3, this is done to counter the swelling effect of ammonium oxalate and shrinking effect of potassium oxalate on the RBCs.
  - *Double oxalates can be used for*
    - Hemoglobin, TLC, RBC count, ESR by
  - *Wintrobe's method and PCV estimation.*
  - *Disadvantages*
    - Leukocytic morphology is not well preserved and hence not suitable for peripheral smear studies
    - The calcium chelated is precipitated in calcium oxalate which is a toxic substance, it is never to be used for blood banking applications.

Making Double Oxalate Bulbs

Prepare double oxalate solution as follows:
1. Potassium oxalate 1.6 g.
2. Ammonium oxalate 2.4 g.
3. Deionized water 100 mL. Mix well, 0.2 mL of the solution will contain 8 mg of the oxalates which prevent clotting of about 3–4 mL of blood.

TRISODIUM CITRATE

Trisodium citrate is used for ESR and some coagulation studies. This too acts by chelating calcium. For ESR, ratio is 1:4; while for coagulation studies ratio is 1:9. 1 part of 3.8% trisodium citrate and 4 or 9 parts of blood respectively.

HEPARIN

Heparin (powder or liquid) acts by inhibiting thrombin and other stages of clotting factor activation.

SPECIAL ANTICOAGULANTS

Special anticoagulants include ACD (acid-citrate-dextrose) used in blood banking and fluoride and oxalate for sugar estimations. Other blood banking anticoagulants are also used.

WHEREVER POSSIBLE, THE NECESSARY TESTS, INVESTIGATIONS AND PREPARATION OF BLOOD FILMS SHOULD BE DONE IMMEDIATELY

If this is not possible, refrigerate the sample at 4°C. Before taking blood from the venous blood containers, invert them gently several times (60) or else unacceptable deterioration in precision may ensue.

ANTICOAGULATED BLOOD STORAGE AND BLOOD CELL MORPHOLOGY

Peripheral Smears

Peripheral smears (anticoagulated or direct blood used and stored at 25 + 5°C). Unfixed smears.
- Up to 60 minutes: No worthwhile notable change
- Up to 3 hours, few changes may be visible
- Up to 12–18 hours: Neutrophils are affected
  - Lobes may get separated
  - Cytoplasmic borders may appear ragged with small intracytoplasmic vacuolation

- Mononuclear cells may show cytoplasmic vacuolation, nuclear disintegration or budding
- RBCs do not change for up to 6 hours at room temperature (25 + 5°C) but longer periods may cause progressive crenation and sphering.

EDTA Blood

On storing EDTA blood, the following changes may occur:

- MCV increases
- Osmotic fragility increases
- Sedimentation rate gradually decreases
- TLC and platelet counts decrease
- Reticulocyte count decreases within 6 hours
- Hemoglobin remains unchanged if the sample does not get infected.

DIAGNOSTIC ALERTS

- Perform all investigations as soon as the blood sample is taken
- Never freeze the sample. On storing the sample at 4°C, the deterioration rate slows down
- Perform all counts within 2 hours of blood collection
- Excessive EDTA in the sample will significantly lower TLC within 1–2 hours
- Leukocytic degenerative changes will affect automated differential counts
- A refrigerated sample must always be brought to room temperature before being used. All samples must be mixed gently, preferably by rotation, for at least 2 minutes before testing.

BLOOD COLLECTION SYSTEM

Whatever be the reason for obtaining blood, in the interest of the patient and your own interest, it is ideal and necessary to use sterile disposable blood collection systems, viz., disposable syringes or the Vacutainers. These are meant for single use and are to be discarded (never to be used again). Relatively new in our country, but established all over the world and being used for decades, is the Vacutainer blood collection system manufactured by *Becton Dickinson* (BD). Other makes/brands are also available

The Vacutainer system consists of a needle, a needle holder and a glass/plastic vacuum tube instead of the syringe barrel and plunger. Once the vein is punctured, the Vacutainer tube appropriately in contact with the needle, the requisite quantity of blood flows automatically into the Vacutainer tube so that the need to pull the plunger out is obviated. Vacutainer is simple to use, quicker, cleaner and safer. It offers— leakproof tubes, standardization of specimen quality at high level, opportunity to rationalize laboratory procedures and innovative, high technology tubes. Appropriate anticoagulants, and other additives are pre-added in appropriate quantities so that all that is required is clean venipuncture. Containers also available for collecting blood from infants with the help of a skin puncture, these are called Microtainers. The blood so collected is adequate for micro or dry chemistries. From a single venipuncture, blood can be collected in separate Vacutainers (for different purposes—EDTA or oxalate-fluoride or citrate Vacutainers) meant for different purposes and very easily identified by the color of their caps. The Vacutainer system is a cleaner system, as blood does not come in contact with atmosphere as it flows straight from the vein through the sterile needle into the sterile tube. The process of transferring blood from syringe to different bulbs is eliminated. Contamination from fallen blood is entirely removed. The incidence of hemolysis is significantly reduced because the major cause of it—the transfer of blood from the syringe to container—is eliminated. In

hematology, because of the instant contact between blood and anticoagulant minimises microclot formation. Furthermore, all Vacutainer tubes are sterile, guaranteeing the biological integrity of the sample—a particularly important factor with ESR determinations and coagulation studies, which can be seriously distorted by microbial growth in the citrate solution. The maximum benefit is derived by the laboratory personnel (by use of Vacutainers) though the physician is assisted only indirectly in the form of quality reports. Also available are Vacutainer culture systems, where blood is injected into the culture media directly without even coming in contact with atmosphere.

Table 24.1 gives color codes for the Vacutainer systems and in all cases different volume containers are available from 2 to 15 mL.

Table 24.1: Vacutainer color codes and their usage

	Stopper color code	*Additive*	*Used for*	*Remarks*
1.	Gray and red	Inert polymer Barrier material	Chemistry tubes	Serum separation
2.	Yellow and red	None	Chemistry	Clot activator tubes
3.	Red	None	Chemistry	Silicone coated interior
4.	Red/orange green	None	Chemistry	—
5.	Yellow	None	Chemistry	Silicon-coated interior
6.	Royal blue	None/heparin/EDTA	Toxicology and nutrition studies e.g., trace elements, heavy metals, etc.	
7.	Brown	Sodium heparin	Lead determination	
8.	Yellow black	Thrombin (NIH)	For stat procedures as thrombin hastens clotting and therefore quick serum separation	
9.	Gray	Sod. fluoride/iodoacetic lithium salt potassium oxalate/potassium oxalate- NaF/EDTA-NaF/thymol NaF	For glucose estimation	
10.	Green	Sodium heparin Lithium heparin Ammonium heparin	For chemistry or cytology	
11.	Blue	Sodium citric acid	For coagulation studies	
12.	Lavender	EDTA	For hematology studies	
13.	Yellow	ACD solution A ACD solution B ACDP solution Alsever's solution	For blood banking For blood banking	
14.	Green	Na heparin	LE cell preparation	Prelabeled

Contd.

Table 24.1: Vacutainer color codes and their usage

	Stopper color code	*Additive*	*Used for*	*Remarks*
15.	Grey/black	Gives citrate Blood a ratio of 1:4	For ESR estimation by Westergren's method	Prelabeled
16.	Blue	Ammonium oxalate and potassium oxalate in a ratio of 6:4	For ESR estimation by Wintrobe's methods	Prelabeled
17.	Yellow	Sodium polyanethol sulfonate 0.35% in 0.85% sodium chloride	For microbiology	Prelabeled

HEMOGLOBIN (HB)

Hemoglobin is the main constituent of the RBCs and carries out the important function of transportation of oxygen from lungs to various parts of the body. To a lesser extent, it transports back carbon dioxide from the body to the lungs. When fully saturated, each gram of hemoglobin holds approximately 1.34 mL of oxygen. The red cell mass of an adult contains approximately 600 g of hemoglobin, capable of carrying 800 mL of oxygen.

HEMOGLOBIN ESTIMATION: SAHLI'S METHOD (SAHLI'S HEMOGLOBINOMETER) (Fig. 24.3)

This is based on conversion of hemoglobin to acid hematin, which has a brown color. Fill hemoglobin tube till 20 mark with N/10 HCl. To this, add blood sucked till the specific mark (20 μL) on the hemoglobin pipette and wait for 5–45 minutes. During this time keep stirring the mixture of acid-blood in the tube. Add distilled water until a match is obtained with the brown glass standard (comparator) provided. Read the lower level of fluid meniscus on g% side of the tube. Report hemoglobin in g/100 mL of blood. If hemoglobin is less than 2 g%, take double the quantity of blood and divide the result by 2. If hemoglobin concentration is extremely high-dilute blood with equal amount of normal saline, take the reading and multiply by 2. This method, however, does not estimate carboxyhemoglobin, methemoglobin and sulph-hemoglobin. Nonhemoglobin substances (protein, lipids) in plasma and cell stroma may influence the color of blood diluted with acid. It is, therefore, not a very satisfactory method.

CYANMETHEMOGLOBIN METHOD

(Drabkin's solution and the standard available from Coral Clinical Systems, Goa)

Drabkin's Reagent

In 1000 mL of deionized water are mixed:

- Potassium ferricyanide: 400 mg
- Potassium dihydrogen phosphate: 280 mg
- Potassium cyanide: 100 mg
- Nonidet (nonionic detergent): 1 mL

This reagent can be stored in a polythene container. Concentrated stock solutions can also be prepared and diluted accordingly when needed.

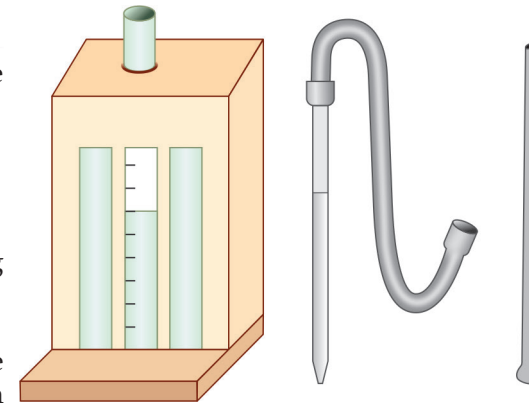


Fig. 24.3: Sahli's hemoglobinometer, middle: Hb pipette, right: Stirrer

Pipette carefully and take care not to discard cyanide solutions into sinks or receptacles containing acid (to prevent formation of hydrocyanic acid). To 5 mL of Drabkin's solution, add 20 µL of blood. Mix well. Read in a photocolorimeter at 540 nm (green filter).

For this procedure, certified standard hemoglobin solution may be obtained from reputable laboratory supply firms. By diluting the known standard hemoglobin solution, a graph (linear) may be obtained by plotting the known Hb concentration against the colorimetric optical density reading so that in future the corresponding hemoglobin value can directly be read off from the calibration curve after knowing the optical density of a particular unknown blood sample.

SHEARD-SANFORD OXYHEMOGLOBIN METHOD

Mix 20 mL of 0.1% sodium carbonate and 0.1 mL of blood or aliquots of these (e.g., 4 mL diluent for 20 µL blood); read optical density in photometer at 540 nm within 30 minutes. Photometer calibration should be based on blood iron determination or oxygen capacity determination.

OTHER METHODS

Alkali Hematin Method

It does not estimate fetal hemoglobin and is no longer used in routine hemoglobinometry.

Gasometric Method

Van Slyke's oxygen capacity method. It is an indirect method, which estimates the amount of hemoglobin from the amount of oxygen it absorbs. This method is very complicated for routine clinical work.

Specific Gravity Method

The normal specific gravity of blood ranges from 1.048 to 1.066. The average for men is 1.057 and for women it is 1.053. From specific gravity of the unknown sample, its hemoglobin is calculated. This is a very rapid and an uncomplicated method and finds its main use in screening potential blood donors for anemia.

Chemical Methods

Obsolete. Hemoglobin is estimated by finding the iron content.

SODIUM LAURYL SULFATE METHOD (AVAILABLE FROM CORAL CLINICAL SYSTEMS, GOA)

This is a KCN-free reagent where SLS substitutes KCN. The color complex formed is SLS-Hb which is read at 540 nm. The greatest advantage being that *all forms of Hb are converted*. The method is relatively free from inter-ferences due to lipemia and presence of WBCs. Linearity is superior to that of Cyanmeth Hb method.

Normal Hemoglobin Values

Men	15.5 ± 2.5 g/dL	14–18 g%
Women	14.0 ± 2.5 g/dL	11.5–16.5 g%
Infants full-term cord blood	16.5 ± 3.0 g/dL	13.5–19.5 g%
Children, 1 year	12.0 ± 1.0 g/dL	11.0–13.0 g%
Children, 10–12 years	13.0 ± 1.5 g/dL	11.5–14.5 g%

According to current WHO specification for males 13.2 g/dL and for females up to 11.7 g/dL Hb are said to be normal. For children from 3 months to puberty, 10.7 g/dL is said to be normal Hb level.

HEMATOCRIT/PACKED CELL VOLUME (PCV)

DEFINITION

Hematocrit is the volume of red cells expressed as a percentage of the volume of whole blood in the sample. The venous hematocrit is almost same as that obtained from a skin puncture. Dried heparin, EDTA or double oxalate are satisfactory anticoagulants.

METHODS

1. Using Wintrobe's tube
2. Using microhematocrit capillaries

Wintrobe's Tube

Fill the Wintrobe's tube till the 100 mark on top with a Pasteur pipette ensuring that there are no air-bubbles in the blood column. Centrifuge this tube for 15 minutes at 3500 rpm (or longer at lower speeds) until packing is complete. After centrifuging, the blood is separated into 3 layers, a column of red blood cells at the bottom, a narrow middle layer—buffy coat of WBCs and platelets and the topmost fluid column of plasma. The percentage of the height of the column of blood occupied by packed red cells constitutes the hematocrit. Roughly, the hematocrit value is three times the hemoglobin concentration.

Sources of error

1. Inadequate mixing of blood
2. Irregularity of the bore of the tube
3. Incomplete packing.

Microhematocrit

This method is in common use in most well- equipped laboratories. Capillary tubes coated with anticoagulant can be filled with blood obtained from finger puncture or from a venipuncture or with blood already anticoagulated. One end of the filled capillary tube is sealed with sealing wax (e.g. Plasticine) or the empty end is sealed with heat. The sealed tube is centrifuged for 3 minutes in a special high-speed centrifuge. By reading the packed cell height and the total height of the entire specimen, the hematocrit can be determined. Special reading devices are available.

Values: If the red cells are of normal size (normocytic), and the red cell count is 5 million, the hematocrit is about 45%.

Men—Range 42–52% Average = 47%

Women—Range 37–47% Average = 42%

INTERPRETATION

Causes of reduced hematocrit—causes of anemia—causes of raised hematocrit—causes of polycythemia.

If packed cell volume has been determined by Wintrobe's tube, one can obtain some more information.

Buffy coat: A buffy coat of thickness 1 mm approximately corresponds to a total leukocyte count of about 10,000. Absent or minimal buffy coat implies leukopenia, a thickness more

than 1 mm implies leukocytosis. In addition, in subleukemic leukemia, a film can be made from the buffy coat where a greater concentration of WBCs will be available, and identification of atypical cells would become easier and less time consuming. Another advantage is for performing LE cell or phenomenon test, for which also WBCs can be picked up from the buffy coat. The platelets form a very thin layer above the white cells, the coat is pinkish white but is of no use clinically, one has to do platelet counts if necessary.

Plasma layer: The topmost layer of plasma can give important clues by observing its color. Its normal color is pale yellow or straw.

Yellow—jaundice; Pink—hemolysis
Creamy white—hyperlipidemia, especially chylomicrons
Brown—methemalbuminemia.

BLOOD CELL COUNTS

WBC

A white cell count (TLC) estimates the total number of white cells in a cubic millimeter of blood. It is important in the diagnosis of disease, especially when accompanied by a differential white cell count.

The diluting fluid: WBC diluting fluid contains a weak acid to lyse the RBCs and a stain for staining the nucleus of WBCs, e.g. Turke's fluid

Glacial acetic acid 1.5 mL
1% aqueous solution of Gentian violet 1.0 mL
Distilled water 98.0 mL
(A pinch of thymol may be added to the diluting fluid to prevent growth of moulds).

Counting Chamber

The chamber normally used for cell counts is the improved Neubauer's chamber which has an area of 9 sq/mm and a depth of 0.1 mm.

Other counting chambers can also be used including the Burker's chamber which has the same area and depth as the improved Neubauer (Fig. 24.4) and the Fuchs–Rosenthal ruled chamber which has the same area but a double depth of 0.2 mm.

Methods

1. Using a WBC pipette (Fig. 24.5) of a hemacytometer, draw well mixed venous blood or capillary blood and fill till the 0.5 mark. Clean the tip of the tube. Now draw WBC diluting fluid till the 11 mark (or to 0.38 mL of diluting fluid add 0.02 mL of blood with a Hb pipette).
2. Mix the fluid and blood mixture gently, avoiding bubbling.
3. Place the coverslip on the counting chamber at the right place.

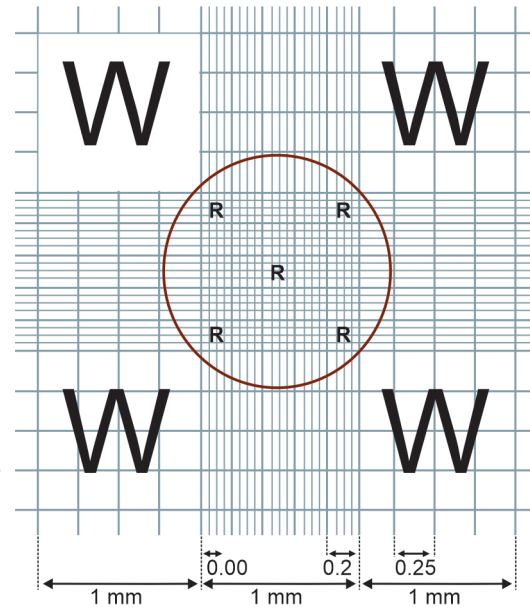


Fig. 24.4: Platelet counting area count the cells in the 25 squares inside the large center square circle

Shake the fluid-blood mixture and transfer the mixture using a fine bore Pasteur pipette on to the counting chamber (called charging the chamber), taking care that the mixture does not overflow. If it does overflow, wash and dry the chamber to be recharged again.

Allow the cells to settle to the bottom of the chamber for 2 minutes. See that fluid does not get dried up (For preventing this, take a petridish, place a wet filter paper at its bottom, now place the charged chamber gently and close off the dish for about 2 minutes).

For counting, clean the under part of the chamber if it was left in the petridish and place it on the stage of the microscope. Using 10x or low power objective, count the WBCs uniformly in the four larger corner squares (as indicated in the diagram). Cells present on the outermost lines should be counted on one side and those present on the line opposite should not be counted.

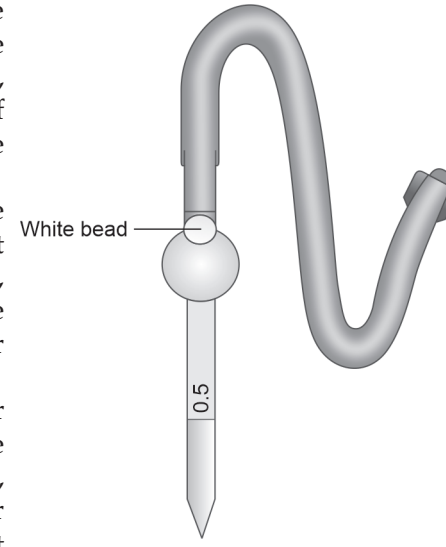


Fig. 24.5: WBC pipette

Calculate the number of cells per cubic millimeter of blood as follows:

$$\frac{\text{Cells counted} \times \text{blood dilution} \times \text{chamber depth}}{\text{Area of chamber counted}}$$

$$= \text{number of cells counted} \times \frac{20 \times 10 \text{ (depth factor)}}{4}$$

= number of cell counted × 50

(Dilution factor is 20 for there is no mixing of cells till first 1 mark of the WBC pipette, hence 0.5 parts of blood are present in 10 parts of the diluting fluid-dilution factor, then, is 10/0.5 = 20).

Falsely high counts occur due to:

1. Blood taken from an area where there was hemoconcentration.
2. Not wiping away the blood on the outside of tip of the pipette.
3. Blood drawn above the mark in the pipette (happens usually when the rubber mouth-piece is too short).
4. Diluting fluid not taken till the requisite mark.
5. Improper mixing.
6. Uneven distribution in the counting chamber.
7. Extraneous material present (yeast, dirt, etc.)
8. Errors in calculation.

Falsely low counts occur due to:

1. Dilution of the blood with tissue fluid due to edema or squeezing.
2. Delay in counting (this does not affect RBCs as much as WBCs, which are reduced by about 15% in 24 hours).
3. Blood not drawn up to the requisite mark.
4. Diluting fluid taken in excess of the requisite mark.

5. Saliva in the mouthpiece running into the upper end of the pipette causing further dilution.
6. Improper mixing.
7. Uneven distribution in the counting chamber.
8. Uniform systematic counting not done.
9. Cells lost through hemolysis (in RBC count for instance).
10. Errors in calculation.
11. Clumping of cells or coagulation of the blood.

Causes of raised and reduced leukocyte counts would be presented with differential leukocyte counts, since raised or lowered total counts are usually accompanied by abnormal differential counts.

Correcting the white cell count for nucleated red cells: From peripheral smear, find out the number of nucleated red cells per 100 WBCs counted.

Calculation

$$\frac{\text{Number of nucleated RBCs}}{100 + \text{number of nucleated RBCs}} \times \text{TLC} = \text{Nucleated RBC}/\text{mm}^3$$

Corrected count = TLC–Nucleated RBC count.

RBCS

Diluting Fluid

This should be isotonic so that RBCs are not hemolyzed. Normal saline can be used but it may cause crenation of the RBCs and allow rouleaux formation.

One can use:

1. Sodium citrate 3 g
   - Formalin 1 mL
   - Distilled water to 100 mL
   - (Cheap and good) or
2. Hayem's fluid
   - Mercuric chloride 0.5 g
   - Sodium chloride 1.0 g
   - Sodium sulfate 5.0 g
   - Distilled water to 200 mL.

(Needs to be made frequently and in hyperglobulinemia one may set precipitation of protein so RBC clumping may occur. Mercuric chloride acts as an antiseptic).

Method

Draw blood to the 0.5 mark in the RBC pipette (Fig. 24.6). Wipe tip clean and draw diluting fluid to the 101 mark. Shake for 3 minutes. Charge the chamber. Count the RBCs using 40x objective in the 80 smallest squares as indicated in the diagram of the chamber.

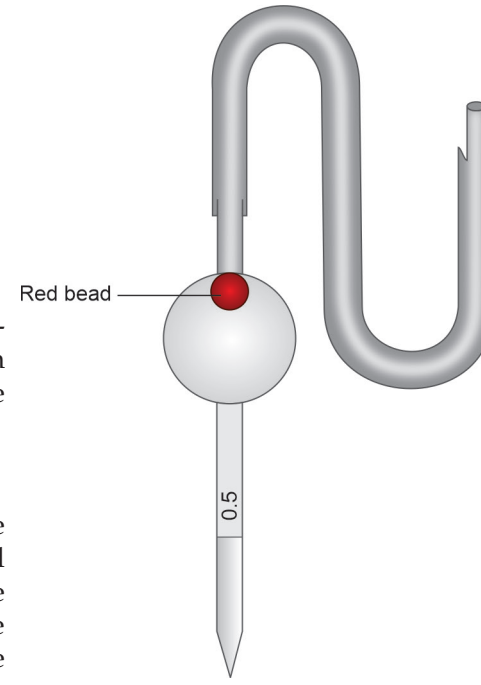


Fig. 24.6: RBC pipette

$$\text{RBC} = \frac{\text{No. of cells counted} \times \text{Dilution factor} \times \text{Depth factor}}{\text{Area counted}}$$

where dilution is 1 in 200, depth is 1/10 mm

$$\text{Area counted is } \frac{80}{400} = \frac{1}{5}\text{mm}^2$$

$$\frac{\text{Number counted} \times 200 \times 10}{1/5} = \text{Number counted} \times 10{,}000$$

Interpretation

RBC counts are low in anemia and high in polycythemia, the causes of these have already been discussed.

PLATELETS

Preferably, use venous blood for platelet counts. Finger prick may cause clumping of platelets. In small children, however, this clumping can be prevented by thinly smearing Vaseline over the area to be punctured (make sure that there has been no clotting of bood).

The blood is diluted in 1% ammonium oxalate stored refrigerated at 4°C which hemolyses the RBCs (prepared by dissolving 1 g of ammonium oxalate in 100 mL of distilled water).

Method

- Fill blood and diluent (in this case 1% ammonium oxalate) as described for the RBC count and using the RBC pipette. If platelet count is low, a WBC pipette can be used instead.
- Charge the chamber with the help of the pipette employed.
- Using 40x objective with reduced condenser aperture, count the platelets in the same squares as indicated for RBC counting.
- Calculate as (if RBC pipette used)

$$\frac{\text{Cells counted} \times \text{blood dilution} \times \text{chamber depth factor}}{\text{Area of chamber counted}}$$

$= N \times 200 \times 5 \times 10$

$= N \times 10000$

However, if a WBC pipette is employed, the appropriate formula and method should be used. Platelet counts are made in the small 5 RBC squares only.

Platelet count = $N \times 20 \times 5 \times 10$ or $N \times 1000$

Normal platelet counts = 1.5–3.5 lakhs/mm^3.

Rees–Ecker Method for Platelet Count

Various components of the diluting fluid used have various functions, e.g. citrate prevents coagulation while formaline fixes the platelets and prevents their clumping together. Here, no attempt is made to lyse RBCs. Platelets are identified by their size, shape and dark color. Brilliant cresyl blue (the dye used) provides the background during cell counting. This dye does not stain the platelets and, therefore, is not essential for the counting procedure.

Diluting Fluid

Consists of:

- Trisodium citrate 3.8 g
- Neutral formaldehyde 0.2 mL
- Brilliant cresyl blue 0.1 g
- Deionized water 100 mL

Dissolve the ingredients in 100 mL volumetric flask, filter, centrifuge, transfer to a well-stoppered bottle and keep at 2–8°C (refrigerate). This fluid if not contaminated will stay good indefinitely. Filter aliquot of the diluting fluid immediately before use.

All Glassware Must be Scrupulously Clean. Dirt or Dust Particles can Resemble and may be Counted as Platelets.

Procedure

- Take 3.98 mL of diluent (freshly filtered) into a test tube.
- Add to the diluent 0.02 mL (20 µl) of well-mixed anticoagulated blood. With the help of a Sahli pipette, wipe out the outer tip of the pipette before dilution. Wash out the contents in the pipette into the diluent tube 3–4 times.
- Immediately mix the diluent with the specimen for at least 5 minutes or so.
- Employ the Sahli's pipette for charging either side of the chamber.
- Keep the charged hemocytometer inside a moist chamber (can be a petridish with a moistened or wet filter paper—on which the chamber can be kept). Let stay for about 15 minutes. This permits the platelets to settle down, and the moistened chamber does not allow evaporation of the fluid.
- Place the hemocytometer on the stage of the microscope, focus the RBC counting area under low magnification. Now move to the corner square of the red cell area carefully to high dry objective.
- Platelets are bluish and must be distinguished from debris. They are oval, round, or comma-shaped, refractile bodies that vary in size normally from 1 to 5 microns.
- Count the platelets in the finely ruled center area (1 mm^2) of each side of the chamber. Take the average counts of two sides.
  (In the new improved Neubauer ruling, there are 25 small squares and each of these contain 16 smallest squares. The area covered by the 25 squares is equal to 1 mm^2.)

$$\text{Platelet count/mL or } mm^3 = \frac{\text{Number of platelets counted} \times \text{Dilution}}{\text{Volume of fluid}}$$

where, volume of fluid for the 1 mm^2 area = 1 × 0.1 = 0.1 mL (mm^3)

Dilution = 200

$$\text{So platelet count/}mm^3 = \frac{\text{Number of platelets counted} \times 200}{0.1}$$

= Number of platelets counted × 2000

Rough Estimation of Platelet Count from Stained Thin Smear

A well-prepared peripheral blood smear can be used to check the results of direct counting. Determine the ratio of platelets to red cells on a thin blood smear used for differential leukocyte count. If the average number of platelets is 8–25 in 10 fields, it is reported to be adequate, and if it is 0–5, it is reported as inadequate.

ERYTHROCYTE INDICES

These can be calculated from:
a. Hematocrit
b. Hemoglobin concentration, and
c. Red cell counts.

1. Mean Cell Volume (MCV)

$$MCV = \frac{\text{Packed cell volume}}{\text{Red cell count per liter}} = 10^{15} fL$$

Normal values
Adults 76–96 fL.
Infants, full-term cord blood Avg. 106 fL.
Children, 1 year 76–87 fL.
Children 10–12 years 76–93 fL.
MCV is reduced in microcytic anemias
MCV is raised in macrocytic anemias

2. Mean Cell Hemoglobin (MCH)

$$MCHC = \frac{\text{Hemoglobin in g/L}}{\text{Red cell count per mL}} \text{ pq}$$

Normal MCH in adults is from 27 to 32 pg
MCH is reduced in hypochromic anemias.

3. Mean Cell Hemoglobin Concentration (MCHC)

$$MCHC = \frac{\text{Hb in g\%}}{\text{PCV}} \times 100 = 31–35 \text{ m\%}$$

This too, is low in hypochromic anemias.

COMPLETE BLOOD COUNT (CBC), BLOOD

NORMAL VALUES

	Normal range, unit	*SI units*
Hematocrit (HCT)		
Adult females	37–47%	0.37–0.47 L/L
Pregnant		
Trimester 1	35–46%	0.35–0.46 L/L
Trimester 2	30–42%	0.30–0.42 L/L
Trimester 3	34–44%	0.34–0.44 L/L
Postpartum	34–44%	0.34–0.44 L/L
Adult males	40–54%	0.40–0.54 L/L
Children		
Newborn	42–68%	0.42–0.68 L/L

Contd.

	Normal range, unit	*SI units*
3 months	29–54%	0.29–0.54 L/L
1 year	29–41%	0.29–0.41 L/L
3 years	31–44%	0.31–0.44 L/L
10 years	34–45%	0.34–0.45 L/L
Hemoglobin (HGB)		
Adult females	12–16 g/dL	7.4–9.9 mmol/L
Pregnant		
Trimester 1	11.4–15.0 g/dL	7.1–9.3 mmol/L
Trimester 2	10.0–14.3 g/dL	6.2–8.9 mmol/L
Trimester 3	10.2–14.4 g/dL	6.3–8.9 mmol/L
Postpartum	10.4–15.0 g/dL	6.4–9.3 mmol/L
Adult males	14.0–18.0 g/dL	8.7–11.2 mmol/L
Panic low level	<5g/dL	<3.1 mmol/L
Panic high level	>18 g/dL	>11.2 mmol/L
Children		
Newborn		
Day 1	15.5–24.5 g/dL	9.6–15.2 mmol/L
Days 2–3	19.0 g/dL	11.8 mmol/L
Days 4–8	14.3–22.3 g/dL	8.9–13.8 mmol/L
Days 9–13	16.5 g/dL	10.2 mmol/L
2–8 weeks	10.7–17.3 g/dL	6.6–10.7 mmol/L
3–5 months	9.9–15.5 g/dL	6.1–9.6 mmol/L
6–11 months	11.8 g/dL	7.3 mmol/L
1–2 years	9.0–14.6 g/dL	5.6–9.0 mmol/L
3–9 years	9.4–15.5 g/dL	5.8–9.6 mmol/L
10 years	10.7–15.5 g/dL	6.6–9.6 mmol/L
11–15 years	13.4 g/dL	8.3 mmol/L
Panic levels	<5 g/dL >18 g/dL	<3.1 mmol/L >11.2 mmol/L
Red Blood Cell (RBC) Count		
Adult females	4.0–5.5 million/μL	4.0–5.5 × 10^{12}/L
Pregnant		
Trimester 1	4.0–5.0 million/μL	4.0–5.0 × 10^{12}/L
Trimester 2	3.2–4.5 million/μL	3.2–4.5 × 10^{12}/L
Trimester 3	3.0–4.9 million/μL	3.0–4.9 × 10^{12}/L
Postpartum	3.2–5.0 million/μL	3.2–5.0 × 10^{12}/L
Adult males	4.5–6.2 million/μL	4.5–6.2 × 10^{12}/L
Children		
Newborn		
Day l	4.1–6.1 million/μL	4.1–6.1 × 10^{12}/L

Contd.

	Normal range, unit	*SI units*
Days 2–8	5.1 million/μL	5.1×10^{12}/L
Days 9–13	5.0 million/μL	5.0×10^{12}/L
2–8 weeks	3.8–5.6 million/μL	$3.8–5.6 \times 10^{12}$/L
3–5 months	3.8–5.2 million/μL	$3.8–5.2 \times 10^{12}$/L
6–11 months	4.6 million/μL	4.6×10^{12}/L
1–2 years		3.6–5.5 million/μL
3 years	4.5 million/μL	4.5×10^{12}/L
4 years	4.0–5.2 million/μL	$4.0–5.2 \times 10^{12}$/L
5 years	4.6 million/μL	4.6×10^{12}/L
6–10 years	4.7 million/μL	4.7×10^{12}/L
11–15 years	4.8 million/μL	4.8×10^{12}/L
Mean Cell Volume (MCV)		
Adults	82–98 μ^3	82–98 fL
Children		
Newborn		
Day 1	106 μ^3	106 fL
Days 2–3	105 μ^3	105 fL
Days 4–8	103 μ^3	103 fL
Days 9–13	98 μ^3	98 fL
2–8 weeks	90 μ^3	90 fL
3 months	82 μ^3	82 fL
4–5 months	80 μ^3	80 fL
6–11 months	77 μ^3	77 fL
1 year	78 μ^3	78 fL
2 years	77 μ^3	77 fL
3 years	79 μ^3	79 fL
4–10 years	80 μ^3	80 fL
11–15 years	82 μ^3	82 fL
Mean Cell Hemoglobin (MCH)		
Adults	26–34 pg	1.61–2.11 fmol
Children		
Newborn		
Day 1	38 pg	2.36 fmol
Days 2–3	37 pg	2.30 fmol
Days 4–8	36 pg	2.23 fmol
Days 9–13	33 pg	2.05 fmol
2–8 weeks	30 pg	1.86 fmol
3 months	28 pg	1.73 fmol
4–5 months	27 pg	1.67 fmol

Contd.

	Normal range, unit	*SI units*
6–11 months	26 pg	1.61 fmol
1–2 years	25 pg	1.55 fmol
3 years	26 pg	1.61 fmol
4–10 years	27 pg	1.67 fmol
11–15 years	28 pg	1.73 fmol
Mean Cell Hemoglobin Concentration (MCHC)		
Adults	31–38%	19.2–23.58 mmol/L
Children		
Newborn		
Day 1	36%	22.34 mmol/L
Days 2–8	35%	21.72 mmol/L
Days 9–13	34%	21.10 mmol/L
2–8 weeks	33%	20.48 mmol/L
3–5 months	34%	21.10 mmol/L
6–11 months	33%	20.48 mmol/L
1–2 years	32%	19.86 mmol/L
3 years	35%	21.72 mmol/L
4–15 years	34%	21.10 mmol/L
White Blood Cell (WBC) Count		
Adult females	4500–11,000/mL	4.5–11.0 × 10^9/L
Pregnant		
Trimester 1	6600–14.100/mL	6.6–14.1 × 10^9/L
Trimester 2	6900–17,100/mL	6.9–17.1 × 10^9/L
Trimester 3	5900–14,700/mL	5.9–14.7 × 10^9/L
Postpartum	9700–25,700/mL	9.7–25.7 × 10^9/L
Adult males	4500–11,000/mL	4.5–11.0 × 10^9/L
Children		
Newborn	9000–30,000/mL	9.0–30.0 × 10^9/L
3 months	5700–18,000/mL	5.7–18.0 × 10^9/L
1 year	6000–17,500/mL	6.0–17.5 × 10^9/L
3 years	5700–16,300/mL	5.7–16.3 × 10^9/L
10 years	4500–13,500/mL	4.5–13.5 × 10^9/L
White Blood Cells Differential		
Granulocytes		
Segmented		
Neutrophil (Segs)	54–62%	0.54–0.62
Adults	3800/μL or mm^3	3800 × 106/L
Children		
Birth	8400/μL or mm^3	8400 × 10^6/L
12 hours	12,100/μL or mm^3	12,100 × 10^6/L

Contd.

	Normal range, unit	*SI units*
24 hours	8870/μL or mm^3	8870 × 10^6/L
1 week	4100/μL or mm^3	4100 × 10^6/L
2 weeks	3320/μL or mm^3	3320 × 10^6/L
1–2 months	2750/μL or mm^3	2750 × 10^6/L
4 months	2730/μL or mm^3	2730 × 10^6/L
6 months	2710/μL or mm^3	2710 × 10^6/L
8 months	2680/μL or mm^3	2680 × 10^6/L
10 months	2600/μL or mm^3	2600 × 10^6/L
12 months	2680/μL or mm^3	2680 × 10^6/L
2 years	2660/μL or mm^3	2660 × 10^6/L
4 years	3040/μL or mm^3	3040 × 10^6/L
6 years	3600/μL or mm^3	3600 × 10^6/L
8–14 years	3700/μL or mm^3	3700 × 10^6/L
15–20 years	3800/μL or mm^3	3800 × 10^6/L
Band		
Neutrophils (Bands)	3–5%	0.03–.05
Adults	620/μL or mm^3	620 × 10^6/L
Children		
Birth	2540/μL or mm^3	2540 × 10^6/L
12 hours	3460/μL or mm^3	3460 × 10^6/L
24 hours	2680/μL or mm^3	2680 × 10^6/L
1 week	1420/μL or mm^3	1420 × 10^6/L
2 weeks	1200/μL or mm^3	1200 × 10^6/L
1 month	1150/μL or mm^3	1150 × 10^6/L
2 months	1100/μL or mm^3	1100 × 10^6/L
4–10 months	1000/μL or mm^3	1000 × 10^6/L
12 months	990/μL or mm^3	990 × 10^6/L
2 years	850/μL or mm^3	850 × 10^6/L
4 years	710/μL or mm^3	710 × 10^6/L
6 years	670/μL or mm^3	670 × 10^6/L
8 years	660/μL or mm^3	660 × 10^6/L
10 years	645/μL or mm^3	645 × 10^6/L
12–14 years	640/μL or mm^3	640 × 10^6/L
15–20 years	620/μL or mm^3	620 × 10^6/L
Eosinophils	1–4%	0.01–0.04
Adults	200/μL or mm^3	200 × 10^6/L
Children		
Birth	400/μL or mm^3	400 × 10^6/L
12–24 hours	450/μL or mm^3	450 × 10^6/L
1 week	500/μL or mm^3	500 × 10^6/L

Contd.

	Normal range, unit	*SI units*
2 weeks	350/μL or mm^3	350×10^6/L
1 month–l year	300/μL or mm^3	300×10^6/L
2 years	280/μL or mm^3	280×10^6/L
4 years	250/μL or mm^3	250×10^6/L
6 years	230/μL or mm^3	230×10^6/L
8–20 years	200/μL or mm^3	200×10^6/L
Basophils	(M) 0.75%	0-0.0075
Adults	40/μL or mm^3	40×10^6/L
Children		
Birth–24 hours	100/μL or mm^3	100×10^6/L
1 week–8 years	50/μL or mm^3	50×10^6/L
10–20 years	40/μL or mm^3	40×10^6/L
Monocytes	2–10%	0.02–0.1
Adults	300/μL or mm^3	300×10^6/L
Children		
Birth	1050/μL or mm^3	1050×10^6/L
12 hours	1200/μL or mm^3	1200×10^6/L
24 hours–1 week	1100/μL or mm^3	1100×10^6/L
2 weeks	1000/μL or mm^3	1000×10^6/L
1 month	700/μL or mm^3	700×10^6/L
2 months	650/μL or mm^3	650×10^6/L
4 months	600/μL or mm^3	600×10^6/L
6–8 months	580/μL or mm^3	580×10^6/L
10–12 months	550/μL or mm^3	550×10^6/L
2 years	530/μL or mm^3	530×10^6/L
4 years	450/μL or mm^3	450×10^6/L
6 years	400/μL or mm^3	400×10^6/L
8–12 years	350/μL or mm^3	350×10^6/L
14 years	380/μL or mm^3	380×10^6/L
16–18 years	400/μL or mm^3	400×10^6/L
20 years	380/μL or mm^3	380×10^6/L
Lymphocytes	20–40%	0.20–0.40
Adults	2500/μL or mm^3	2500×10^6/L
Children		
Birth–12 hours	5500/μL or mm^3	5500×10^6/L
24 hours	5800/μL or mm^3	5800×10^6/L
1 week	5000/μL or mm^3	5000×10^6/L
2 weeks	5500/μL or mm^3	5500×10^6/L
1 month	6000/μL or mm^3	6000×10^6/L

Contd.

	Normal range, unit	*SI units*
2 months	6300/μL or mm^3	6300 × 10^6/L
4 months	6800/μL or mm^3	6800 × 10^6/L
6 months	7300/μL or mm^3	7300 × 10^6/L
8 months	7600/μL or mm^3	7600 × 10^6/L
10 months	7500/μL or mm^3	7500 × 10^6/L
12 months	7000/μL or mm^3	7000 × 10^6/L
2 years	6300/μL or mm^3	6300 × 10^6/L
4 years	4500/μL or mm^3	4500 × 10^6/L
6 years	3500/μL or mm^3	3500 × 10^6/L
8 years	3300/μL or mm^3	3300 × 10^6/L
10 years	3100/μL or mm^3	3100 × 10^6/L
12 years	3000/μL or mm^3	3000 × 10^6/L
14 years	2900/μL or mm^3	2900 × 10^6/L
16 years	2800/μL or mm^3	2800 × 10^6/L
18 years	2700/μL or mm^3	2700 × 10^6/L
20 years	2500/μL or mm^3	2500 × 10^6/L
Platelets		
Adults	150,000–400,000/μL or mm^3	150–400 × 10^9/L
Panic/levels	<30,000/μL or mm^3 >l,000,000/μL or mm^3	<30 × 10^9/L >1000 × 10^9/L
Children		
Newborn	100,000–300,000/μL or mm^3	100–300 × 10^9/L
3 months	260,000/μL or mm^3	260 × 10^9/L
1–10 years	250,000/μL or mm^3	250 × 109/L
Panic/levels	<20,000/μL or mm^3	<20 × 10^9/L
	>1,000,000/μL or mm^3	>1000 × 10^9/L

COMPLETE BLOOD COUNT (CBC)

CBC includes—TLC, DLC, RBC count, hemoglobin, hematocrit, RBC indices, platelet count and a peripheral smear examination.

ERYTHROCYTE SEDIMENTATION RATE (ESR)

This is the rate at which erythrocytes sediment on their own weight when anticoagulated blood is held in a vertical column, it is expressed as the fall of RBCs in mm at the end of first hour (starting point—when the tube or pipette was filled with blood).

METHODS

Westergren's Method

1. Westergren's pipette (open at both ends) is about 30 cm long with a bore diameter of about 2.5 mm (Fig. 24.7).
2. The lower 20 cm are marked from 0 (top) to 200 (bottom).

3. Anticoagulant used is 3.8% trisodium citrate solution. One part of anticoagulant is added to 4 parts of blood.
4. The pipette accepts about 1 mL of blood. Fill the pipette by sucking till the 0 mark and clamp it vertically in the Westergren's rack.
5. Read the upper level of red cells exactly after 1 hour.

This is a better method than Wintrobe's since the reading obtained is magnified as the column is lengthier.

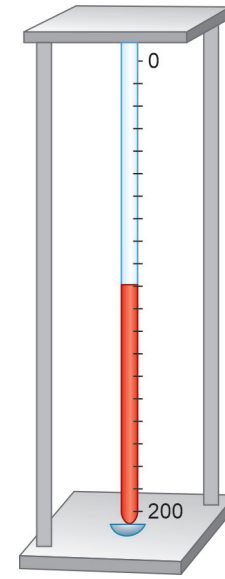


Fig. 24.7: Westergren's ESR pipette with stand

Normal Values

Males—0–5 mm at the end of 1st hour. Females—0–7 mm at the end of 1st hour.

WINTROBE'S METHOD

1. The Wintrobe's tube (Fig. 24.8) is about 11 cm long, bore diameter is 2.5 mm and the bottom 10 cm are graduated.
2. Graduations are from zero (top) to hundred (bottom) for ESR and zero (bottom) to hundred (top) for PCV.
3. EDTA blood is used and the tube is filled till zero mark on top with the help of a Pasteur pipette.
4. Set it up vertically and read exactly after 1 hour.
5. As has already been said that this tube can also be used for PCV estimation.

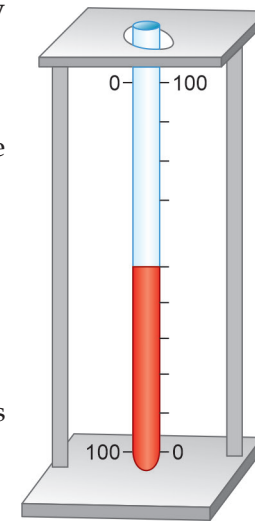


Fig. 24.8: Wintrobe's ESR tube with stand

Normal Values

Males—0–9 mm at the end of 1st hour. Females—0–20 mm at the end of 1st hour.

Microsedimentation (Landau) Method

Capillary blood can be taken.

Materials Required

1. 5.0 g/dL sodium citrate solution.
2. Landau pipette: This pipette resembles RBC pipette. It is graduated from 0 to 50 mm.
3. Landau pipette stand.
4. Suction device for drawing blood into the pipette.
5. Capillaries for blood collection.

Procedure

1. Attach Landau pipette to the suction device.
2. Draw 5.0 g/dL citrate up to first line on the stem.
3. Now draw blood by suction device up to second mark on the stem (avoid air bubbles).
4. Wipe excess blood on the external side of the pipette.
5. Draw citrate solution and blood into the bulb of the pipette. Mix the contents thoroughly.
6. Force back the mixture into the stem of the pipette.
7. Set the upper level of the mixture of the zero mm mark at the top.

8. Detach the suction device.
9. Place the pipette in vertical position on the stand, set time to one hour.
10. Note the reading (the distance red cells have fallen or the extent of plasma column) after one hour.

Normal Values

Male: 0–5 mm after 1st hour Female: 0–8 mm after 1st hour

ZETA SEDIMENTATION RATE (ZSR)

The zeta sedimentation rate (ZSR) is performed using a special small-bore capillary tube that is filled with blood and span for 3-4 minutes in a centrifuge called Zetafuge® (Beckman Coulter). This centrifuge alternately compacts and disposes the RBCs under standardized centrifugal force. The tube is then read on a special reader to obtain a value called the Zetacrit which represents the percentage of sedimented erythrocytes. The Zetacrit value is divided into the hematocrit value (also a percentage) and the result in the ZSR expressed as percentage.

The ZSR's advantages are that it is rapid, correct for anemia, and requires only a small blood sample which is desirable for pediatric patients. However, a special centrifuge and reader are needed to perform the test.

Many other automated systems/devices are also available.

SOURCES OF ERROR FOR ANY ESR METHOD

1. Improper anticoagulant.
2. Tube not vertical, an inclination of 3° raises ESR by almost 30%.
3. Dirty tube.
4. Bubbles caused by too vigorous mixing.
5. Hemolysis may modify ESR.
6. Prolonged storage of blood after withdrawing it, the test should be performed within 3 hours.
7. Pipette/tube kept on a vibrating surface (vibration prevents rouleaux formation).

INTERPRETATION OF ESR

The value of ESR is that it indicates the possible presence of organic disease, or to follow the course of disease. Its main use is as a prognostic tool. It is used as a diagnostic criterion (minor) in rheumatic fever only.

Rapid ESR is Found in

1. In any chronic infection, e.g. tuberculosis (maximum in miliary tuberculosis), has prognostic value.
2. Any extensive inflammation, cell destruction or toxaemia.
3. Pregnancy, after the second month.
4. Puerperium, returns to normal within 2 months.
5. Active myocardial infarction (rapid rise).
6. Acute myocardial infarction (rapid rise).
7. Active rheumatoid arthritis (not much elevated in osteoarthritis).
8. Nephrosis (low blood albumin, anemia).
9. All types of shock.
10. Active syphilis (moderate acceleration).

11. Postoperative states (for variable periods).
12. Any active infectious disease, acute or chronic.
13. Salpingitis, appendicitis (often normal), due to absorption of purulent necrotic material.
14. Infected, necrotic or malignant tumors.
15. Liver disease (depends upon blood proteins).
16. Menstruation (slight acceleration).

Slow ESR is Usually Seen in

1. Newborn infants.
2. Polycythemia.
3. Congestive heart failure.
4. Allergic states.
5. Sickle cell anemia (poikilocytosis).

FACTORS THAT PLAY A ROLE IN ESR

1. **Plasma factors**
   - An accelerated ESR is favored by elevated levels of fibrinogen, and to a lesser extent, of globulins (α and β globulins are more effective than γ globulin).
   - These plasma factors cause increased formation of rouleaux which due to more weight sediment more rapidly than do single cells.
   - Albumin retards sedimentation.
   - Extreme increase in plasma viscosity slows down ESR.
   - Cholesterol accelerates and lecithin retards the ESR.
2. **Red cell factors**
   - Anemia is responsible for accelerated ESR. The change in erythrocyte-plasma ratio favors rouleaux formation.
   - Microcytes sediment more slowly and macrocytes somewhat more rapidly than normocytes. The sedimentation rate is directly proportional to the weight of the cell aggregate and inversely proportional to the surface area.
   - Poikilocytosis retards ESR because abnormal shape hampers rouleaux formation.
3. **Anticoagulants**
   - Sodium citrate and EDTA do not affect ESR but oxalates and heparin may.

Stages in ESR

1. First 10 minutes—is the period of aggregation. Rouleaux formation occurs at this stage and sedimentation is slow.
2. Next 40 minutes—is period of fast settling, during this period rate of fall is constant.
3. Last 10 minutes—is the final period of packing.

INTERFERING FACTORS

1. The blood sample should not be allowed to stand for more than 2 hours before the test is started because rate will increase.
2. In refrigerated blood the sedimentation rate is greatly increased. Refrigerated blood should be allowed to return to room temperature before the test is performed.
3. Factors leading to reduced rates:
   - High blood sugar
   - High albumin level
   - High phospholipids

- Decreased fibrinogen level of the blood in newborns
- Certain drugs (see below)

4. Drugs
   a. That increase ESR levels
      - Dextran
      - Methyldopa
      - Methysergide
      - Oral contraceptives
      - Penicillamine
      - Theophylline
      - Trifluperidol
      - Vitamin A
   b. Those that decrease levels
      - Ethambutol
      - Quinine
      - Salicylates
      - Drugs that cause a high blood glucose level (cortisone and ACTH)

BLOOD FILM EXAMINATION

PREPARATION OF A THIN BLOOD FILM

A thin blood film is made by spreading a drop of blood evenly across a clean grease-free slide, using a smooth edged spreader.

Making of Spreaders (Fig. 24.9)

- Select a slide which has smooth edges
- Using a glass cutter and a ruler, mark off 4 equal divisions, each measuring 19 mm
- Break off at each division to give 4 spreaders
- Ready made spreaders are available.

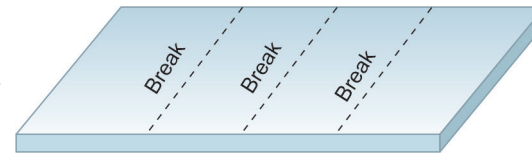


Fig. 24.9: Making a spreader

For anemic blood, a rapid smearing is needed; whereas for thick concentrated blood, smearing should be done slowly. A well-spread smear shows no lines extending across or downwards through the film and the smear should be tongue shaped (Fig. 24.10).

Making Thick Smears

While the thin smears are used for describing blood cells, the thick smears are used for detecting malarial parasites and microfilariae. A large drop of blood is taken on the center of a slide and with the aid of a needle or slide corner spread the drop over ½ an inch square area. When dry, the thickness should be such that printed matter can be seen through it.

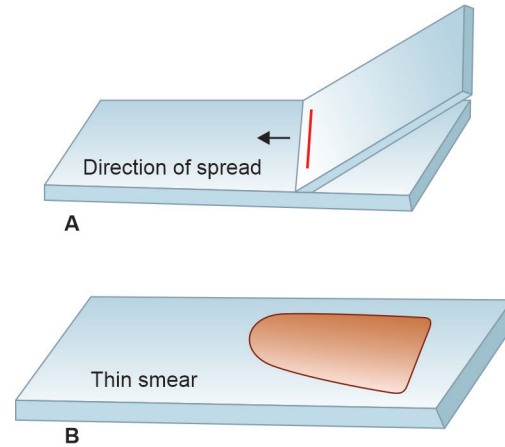


Fig. 24.10: (A) Direction of spread; (B) A thin peripheral blood smear

FIXING OF BLOOD FILMS

Before staining, the blood films need to be fixed with acetone-free methyl alcohol for ½ to 1 minute in order to prevent hemolysis when they come in contact with water while staining them with aqueous (water-based) stains or when water has to be added subsequently. Alcohol denatures the proteins and hardens the cell contents. For Wright's stain and Leishman's stain, no prefixation is required as these contain acetone-free methyl alcohol; but for Giemsa's stain, prefixation is a must because the alcohol content is only 5% in the ready-to-use stain.

STAINING OF BLOOD FILMS

Blood cells have structures that are acidophilic and some basophilic structures, so they vary in their reaction (pH). The nuclei are basophilic and stain blue. The highly basophilic (acidic) basophil granules also stain blue. Hemoglobin (being basic) stains acidophilic or red.

Stains that are made up of combinations of acid and basic dyes are called Romanowsky stain and various modifications are available, e.g. Wright's, Leishman's, Giemsa's, and Jenner's stains. Most use methylene blue as the basic stain, though toluidine blue is used in some. Most use eosin as the acid stain, though Azure I and Azure II are also used.

The dried film can stay for a couple of days in hot dry weather, but gets bad if they are not fixed in hot and humid climate.

It is best to use neutral distilled water for diluting the stain. Stale distilled water becomes acidic after absorbing CO_2 from atmosphere. If the distilled water is alkaline RBCs stain a dirty bluish green color, the parts of WBC which should stain blue will be slightly purplish, the granules of eosinophils bluish or greenish instead of pink and granules of neutrophils overstained. If the water is acidic RBCs stain bright orange and nuclei of the white cells a very pale color.

The ideal pH is 6.8 and in order to maintain this buffered distilled water is used. Buffer water is a solution which tends to keep its original pH even on addition of small amount of alkali or acid. (Buffer tablets ready for use, to be dissolved in distilled water).

Buffer Solution Used in the Laboratory

- Solution No. I
  - NaOH (sodium hydroxide) 8 g.
  - Distilled water 1000 cc.
- Solution No. II
  - KH_2PO_4 (Potassium dihydrogen phosphate)
  - 27.2 g.
  - Distilled water 1000 cc.

Take 23.7 cc of solution I, add to it 50 cc of solution II, add 20 cc of the above mixed solution to 1000 cc of distilled water. This has a pH of 6.8.

Stain Preparation and Staining

- Wright's stain
- Wright's stain (powder) 0.2 g. Acetone free methyl alcohol 100 cc Let stand this solution for a few days.
- If the WBC granules do not stand out clearly, try out a 0.25 or 0.3% solution.

Method

Cover the slide with stain for 1–2 minutes taking care that it does not dry on the slide. Now dilute this with equal amount of buffer water (if the stain is ripe, a scum or film with a metallic sheen will form on the surface of the diluted stains on the slide). The diluted stain is allowed to act for 3–5 minutes and then flooded off with buffer or tap water. The stain should never be poured off or a precipitate of the stain will be deposited on the slide. Should this occur, it can sometimes be removed by flooding the slide with undiluted stain for 10–15 seconds and then washing it off again by flooding the slide once more with buffered water.

Leishman's Stain

- Powdered Leishman's stain 0.15 g.
  - Acetone-free methyl alcohol 133 mL.

All the stain should be dissolved (better if the stain crystals are well ground before), keep the stain in a glass stoppered bottle. Do not filter.

Method

Like that for Wright's stain but with double dilution of the buffer water; (i) Pour few drops (about 8) on the slide. Wait for 2 minutes, (ii) Add double the amount (16 drops) of buffered water. Mix by rocking and not by blowing and wait for 7–10 minutes, (iii) The stain is flooded off with distilled water and this should be complete in 2–3 seconds. Longer washing will remove stain, and (iv) Stand in a rack to drain and air dry. A fan will expedite the process.

Giemsa's Stain

- Giemsa powder 0.3 g
  - Glycerine 25.0 mL
  - Acetone free methyl alcohol 25.0 mL.

This makes stock solution and before use it has to be diluted by adding 1 mL (stain) to 9 mL of buffered distilled water.

Method

The blood film is fixed with methyl alcohol for 3–5 minutes and dried. Pour on diluted stain and keep for 15 minutes or longer. Wash off with tap water or neutral distilled water and dry.

Staining of Thick Films

Thick films have to be dehemoglobinized before staining with one of the previously mentioned stains. The slide is kept in distilled water for 10 minutes, then taken out, dried and stained with any of the stains already mentioned. They must not be fixed before staining, or the water will not hemolyze the cells. The stains commonly used are Field's stain and Simeon's stain.

Field's Stain

- Field's stain A
  - Methylene blue 0.8 g
  - Azure I 0.5 g
  - Disodium hydrogen phosphate (anhydrous) 5.0 g

- Potassium dihydrogen phosphate anhydrous 6.25 g
- Disttiled water 500 mL

- Field's stain B
  - Eosin (yellow eosin, water soluble) 1.0 g
  - Disodium hydrogen phosphate (anhydrous) 5.0 g
  - Potassium dihydrogen phosphate (anhydrous) 6.25 g
  - Distilled water 500 mL.

Grind all solids well and dissolve in the said solvent, keep the stains for 4 hours for ripening and filter before use. Keep the stains in covered jars. The depth of the solution should be about 3 inches, the level should be maintained by adding more of the stain solution.

Method

1. Dip the film for one second in solution A.
2. Remove from solution A and immediately rinse by waving very gently in clean water for a few seconds, until the stain ceases to flow from the film and the glass of the slide is free from stain.
3. Dip for one second in solution B.
4. Rinse by waving gently for 2–3 seconds in clean water.
5. Place vertically in a rack to drain and dry.

Simeon's Modification of Boye's and Sterenal's Method

This stain can be used instead of Leishman's or Wright's stain when methyl alcohol is not available to prepare them.

Solution I

- Eosin pure 1 g
- Distilled water 1000 mL

Solution II

a. Medicinal methylene blue 1 g
   Distlilled water 75 mL } Dissolve completely

b. Potassium permanganate 1.5 g
   Distilled water 75 mL } Dissolve completely

1. Mix (a) and (b) in a flask. A massive precipitate is formed.
2. The flask is kept in a water bath at boiling point for half an hour during which time the precipitate redissolves.
3. Filter. The stain is now ready for use, it needs no further dilution.

Method for Staining Thin Films

1. Fix the smear by immersion into rectified spirit—1 minute.
2. Rinse with tap water—4 seconds.
3. Immerse into solution I—10 seconds.
4. Rinse with tap water—4 seconds.
5. Immerse into solution II—15 seconds.
6. Rinse with tap water—4 seconds.
7. Immerse again into solution I—5 seconds.
8. Rinse with tap water—4 seconds.
9. Allow to dry in an upright position.

Procedure for Staining Thick Smears

1. Dehemoglobinize by immersion into tap water, if necessary.
2. Immerse in Sterenel's blue (solution II)—6 seconds.
3. Wash in tap water.
4. Immerse in eosin solution (solution I)—12 seconds.
5. Wash in tap water, allow it to dry in air.
   Examine under microscope.
   The stains are useful for screening purposes.

Mounting and Preservation of Films

Unstained films cannot be preserved well. Due to hardening of plasma, they do not stain well after some time. Stained films if left unmounted tend to fade away rapidly. Canada balsum should not be used as it decolorises the smear. Gurr's neutral mounting medium is quite satisfactory. Use only thin coverslips for mounting.

RAPID DIAGNOSTICS

AUTOMATION IN HEMATOLOGY

Coulter Principle

The *Coulter principle* states that particles pulled through an orifice, concurrent with an electrical current, produce a change in impedance that is proportional to the size of the particle traversing the orifice. The Coulter principle was named for its inventor, Wallace H. Coulter.

Wallace was an electrical engineer by training with a passion for radio technology. During the second world war, Wallace joined the US Navy. While working on a technique to detect submarines using sonar, he frequently detected large echos where no submarines were operating. In an attempt to determine the source, Wallace lowered a series of small bottles with remote trap doors to various depths. The bottles were constructed such that the remote door could be opened and shut at predetermined depths, filling the bottle with seawater from that depth. The source of the false echos turned out to be high concentrations of plankton. In order to count the number of plankton cells per milliliter of seawater accurately and reproducibly, Wallace created a device that would become the basis for the Coulter principle.

The device consisted of a dual chambered container whose two sides were separated by a thin membrane. A small hole in the membrane called an aperture was the only connection between the two chambers. Electrodes from a battery were placed in the chambers, positive on one side and negative on the other. An ohm meter was connected to the circuit so as to measure the resistance to the flow of current (impedance) from one electrode, through the orifice, and to the other electrode. Both chambers were filled with seawater from the trap bottles. Then one of the two chambers was partially drained, forcing seawater to flow from the opposite chamber, through the orifice to balance the level of liquid in the two sides. As the seawater passed through the orifice so did the plankton cells, which created momentary changes in impedance that were seen on the ohm meter. By counting the number of impedance pulses per unit of seawater, Wallace's device was able to count the number of plankton particles.

This technology found commercial success in the medical industry where it revolutionized the science of hematology. Red blood cells, white blood cells and platelets

make up the majority of the formed elements in the blood. The average salinity of human blood is very close to that of seawater, and mixture of salt (NaCl) and water with the same salinity as seawater is said to be isotonic with whole blood. When whole anticoagulated human blood is diluted with isotonic saline, the Coulter principle can be applied to count and size the various cells that make up whole blood. The first commercial application of the Coulter principle to hematology came in 1954 with the release of the Coulter Counter Model A (developed by Wallace and brother Joseph R Coulter). Within a decade, literally every hospital laboratory in the United States had a Coulter Counter, and today every modern hematology analyzer depends in some way on the Coulter principle.

Basics of Hematology Analyzers in a Nutshell

Hematology cell counters continue to provide an ever-broader scope of capabilities. Technologies that were leading edge a few years ago, such as reticulocyte enumeration, are now routine. Methods that heretofore required much manual manipulation—such as CD4 counts—can now be incorporated as part of the random-access CBC specimen stream on instruments such as the Abbott Cell-Dyn series. Food and Drug Administration approval of quantitative nucleated red blood counts on several instruments now permits automated handling of patients with a variety of pathologic states.

For 25 years, the holy grail in the automated counting of the WBC differential has been the enumeration/quantification of immature granulocytes. This debate continues with clinical colleagues who insist they must have a manual differential because they want to know if "bands" are numerous. It does not face them that study after study demonstrate that the "band count" is terribly imprecise and non-reproducible. At least one manufacturer has submitted applications to the FDA for clinical use of the "immature granulocyte" channel. This advance has great potential for the precise and accurate quantitation of immature granulocyte forms (the collective total of promyelocytes, myelocytes, and meta- myelocytes). Ironically, the clinical significance of automated immature granulocyte counts is difficult to measure at present, since the existing literature is heavily weighted toward only band counts and not extended immature granulocyte counts. We do hope to see these immature granulocyte counts take hold and, finally, eliminate the use of the manual band count.

Bayer's reticulocyte hemoglobin measurement is useful in the early diagnosis of iron deficiency and in monitoring response to treatment.

Another interesting new channel is hematopoietic progenitor cells, or HPCs, available on the Sysmex XE-2100. In some settings, this will permit stem cells to be quantitated (for example, in an apheresis product) without requiring a direct CD34 study on a flow cytometer. This study is based on differential membrane lipid content. HPCs have lower membrane lipid content than mature leukocytes and are preserved after treatment with a lysing agent.

With increasing routine automation of assays that previously required the use of flow cytometers, we may see flow cytometers redirected to more in-depth analyses of cell structure and function—the emerging field of cytomics.

The rate-limiting step on the introduction of new diagnostic modalities is no longer a matter of how quickly the technology can be developed, licensed, and deployed. Far more important is how quickly medical practitioners embrace the new technologies and incorporate them into their routines.

Those selecting hematology instruments can no longer base their decisions solely on the lowest-price instrument. Medical considerations should and may dominate. Operational

considerations may be paramount—reliable, high- throughput, easy-to-use instrumentation may be more crucial than having all the newest parameters on a more difficult-to-use instrument. The fiscal effect of eliminating flow cytometry for high-volume studies, such as CD4 or CD34, may outweigh a higher cost-per-test on CBCs.

COULTER MAXM AND MAXM AL HEMATOLOGY FLOW CYTOMETRY SYSTEMS (FIG. 24.11)

The MAXM is the easiest hematology system to learn and operate. It features walkaway operation, positive patient identification, automatic calibration, auto-probe wipe, single-operator interface and continuous computer monitoring of system performance. Best of all, the MAXM requires no routine daily maintenance. Your staff is free to handle more complex tasks.

The optional Autoloader allows walkaway operation. Load 25 bar-coded samples and then just walk away. The MAXM automatically analyzes both patient samples and controls-providing automatic printouts of the finished reports at a throughput of up to 75 samples per hour-unsupervised.

Coulter MAXM AL together with Coulter STKS and the Coulter GEN.STM System offer the ONLY fail-safe sample management system with positive patient ID and monitoring of sample integrity both pre- and post-sampling. This means peace-of mind for you, no reports incorrectly distributed because of short samples and no mix-up in the identity of the patient.

Instrument Specifications

20 Parameters

- White blood cell count
- Lymphocyte % and #
- Monocyte % and #
- Neutrophil % and #
- Eosinophil % and #
- Basophil % and #
- Red blood cell count
- Hemoglobin concentration
- Hematocrit
- Mean corpuscular volume
- Mean corpuscular hemoglobin
- Mean corpuscular hemoglobin concentration
- Red cell distribution width
- Platelet count
- Mean platelet volume.

Fig. 24.11: Coulter MAXM

- Three-dimensional VCS technology provides the highest sensitivity, specificity and efficiency in abnormality detection
- Compact, benchtop analyzer saves valuable lab space
- 75 samples-per-hour throughout maximizes productivity
- Detailed reports and histograms for operator review
- Automatic calibration and zero-routine-maintenance maximizes uptime
- Data management system stores up to 5,000 patient records
- Closed vial sampling, automatic cap piercing and probe wipe minimize biohazards
- Walkaway automation frees up valuable operator time
- Positive patient ID makes sample tracking easy
- No routine maintenance
- Built-in-quality Assurance ensures accuracy

Throughput

- 75 samples per hour
- 30 samples per hour for Retics

Sample Requirements

- 185 µL primary mode
- 125 µL secondary sample mode
- 50 µL predilute mode

Patient Result Storage

- 1,000 sets plus sample analysis screen displays
- 5,000 sets plus all sample analysis screen displays for Retic units

Bar Code Symbology

- Code 39®
- Codabar
- Interleaved 2 of 5
- Code 128

0–24 Hours Sample Stability

- Near-native state analysis of WBC using four reagents that are safe to use and discard.
- Printouts via standard graphics printer with optional color kit, or single ticket printer

High Efficiency through Comprehensive Flagging

Instrument-defined suspect abnormalities (User-defined abnormalities)

- Definitive flags
- High and low lab action limits
- RBC morphology gradient

EXAMINATION OF A BLOOD FILM

Method

1. *Mount:* Cover the slide using a neutral mounting medium.
2. *Low power field examination:* Look for:
   - Quality of film
   - Number, distribution and staining of WBCs.
   - RBCs examination, select an area where they just touch each other without overlapping, i.e. between tail and body of the film.
3. *High power field examination:* Assess RBC
   - Size
   - Shape
   - Hemoglobin concentration
4. *Oil immersion examination:* Assess atypical cells and note fine details, e.g. inclusion bodies.

Always Note

RBCs

- *Size:* Normocytes, microcytes, macrocytes, anisocytosis (variation in size).
- *Shape:* Abnormal shape oval, pencil, tear, pear, oat and sickle-shaped cells, fragmented cells, target cells, spherocytes, crenated cells, burr cells, acanthocytes, stomatocytes (poikilocytes).

- *Hemoglobin:* Normochromic, hypochromic.
- *Immature forms:* Polychromatic, stippled or nucleated red cells.
- *Inclusion bodies:* Howell–Jolly bodies, Cabot rings, Pappenheimer bodies, malarial parasites, etc.
- *Arrangement:* Autoagglutination, excess rouleaux formation.

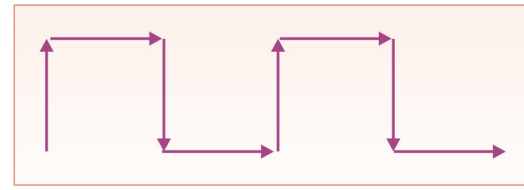
WBCs

- *Number:* Normal, increased, decreased.
- *Abnormal or immature forms:* Immature forms, hypersegmented macropolycytes, abnormal forms.

Platelets

- *Number:* Normal, increased, decreased.
- *Form:* Abnormalities of size and shape, present in groups or lying scattered.

Differential Leukocyte Count (DLC)



For differential leukocyte counts, choose an area where the morphology of the cells is clearly visible. Ensure that there is no tailing of the WBCs or else a false DLC may be obtained. Do differential count by moving the slide as shown in order to include central and peripheral areas of the smear.

While doing DLC, look for vacuolation, toxic granulation, size and maturity of the WBCs. Count at least 100 cells and give percentage of the cells seen. Counting becomes easier if 100 squares are made on a paper and the letters P for neutrophil, L for lymphocyte, M for monocyte, E for eosinophil and B for basophil can be entered in each square. Another easier way is using laboratory cell counter (Fig. 24.12).

BONE MARROW EXAMINATION

Bone marrow can be obtained by using Salah's, Klima's or Jamshidi's marrow aspiration needles. Not more than 0.2 mL of bone marrow should be taken out at one time. If blood or dry taps have occurred on 2 different occasions, a trephine biopsy should be performed.

For aspiration of bone marrow, Klima's needle is better as the guard has no chances of getting slipped and hence dangers of puncturing substernal structures are less.

Before entering the site of puncture, take all aseptic precautions, i.e. cleaning with spirit, iodine and spirit again. The various sites for obtaining bone marrow are:

In adults:

- Sternal aspiration.
- Anterior iliac crest.

Fig. 24.12: Nine-unit laboratory cell counter

- Vertebral spinous processes.
- Posterior superior iliac spine.

In children:

- Tibia, superior medial surface of the tibia, inferior to the medial condyle and medial to the tibial tuberosity.
- Posterior iliac crest.
- Calcaneum.

Bone Marrow Examination and Reporting

Marrow Film Preparation

Delay in marrow film preparation should not be there. Get rid of blood by using a capillary pipette leaving the greyish marrow particles behind. Make a smear as has been described for peripheral blood. The smear should be 3–5 cm long and should not be more than 2 cm wide. The particles should be dragged behind but not squashed. A trail of cells is left behind. Criteria of a good preparation—presence of both particles and free marrow cells in the smear.

Imprints

Another way is imprint method. The marrow particle is picked and transferred immediately to a slide and made to stick to it by a gentle smearing motion. The slide is air dried rapidly by waving.

Crush Preparation

Marrow particles in a small drop of aspirate are placed on a slide near one end. Another slide is carefully placed over the first. Slight pressure is exerted to crush the particles, and the slides are separated by pulling them apart in a direction parallel to their surfaces. Dry the smears immediately. The appearance of fat (irregular holes) on the smear implies that marrow has been obtained.

1. Good fixation is essential.
2. Any of the previously mentioned stains can be used for staining bone marrow films.

Examination

The examiner should be informed of the clinical picture before he or she examines the marrow films. An impression can be formed by examining various fields on the different stained slides prepared. Start with 10x through 40x, to oil immersion, now actually make a differential count of large number of cells (300–1000) and calculate the percentage of each type of cell.

The procedure given below is recommended for studying the marrow films:

1. Naked eye inspection of the slides to select the smear containing particles.
2. With 10x objective, survey the particles whether they are normoplastic, hypoplastic or hyperplastic.
3. Select a cellular area (usually in the tail portion of the film around particles), study the cytologic details by high power—40x and oil immersion—100x objectives.

Note the undermentioned points:

Cellularity: Normo/hypo/hyperplastic marrow particles. Cellularity is better defined by studying histologic sections of aspirated particles, though crude estimates can be given on films. The normal cellularity of the marrow varies with age being more in infants and least in elderly individuals.

Next look for reaction of erythropoiesis, whether it is normoblastic, megaloblastic or micronormoblastic. Look for maturity of leukopoietic cells. The M:E ratio is based on a count of 500–1000 marrow cells. In the normal adult the reaction is normoblastic, leukopoietic maturity is normal and ME ratio is about 3 or 4:1. The ME ratio at birth is 1.85:1; during the first two weeks, it reaches its peak of 11:1. It then gradually drops to the (years 1–20) average of 3:1.

Look for megakaryocytes, their number, size, nuclear

Megakaryocytes with budding or cloud-like appearance at the periphery are functioning ones. In addition, if suspected, look for metastatic cells, increased number of normal or abnormal (myeloma) plasma cells, cells containing unduly large amounts of lipid, carbohydrate, etc.—storage disease. One may also see hemoparasites in the marrow especially LD bodies of kala-azar.

Bone Marrow Aspiration Analysis

- *Normal Values:* Red marrow contains connective tissue, fat cells, and hematopoietic cells. Yellow marrow contains connective tissue and fat cells. Interpretation of cell count and histopathology by a hematologist, pathologist, or oncologist is required.
- *Response to staining:* Iron stain for hemosiderin: 2+.
- *Periodic acid-Schiff (PAS) glycogen reactions:* Negative.
- *Sudan black B (SBB) granulocyte:* Negative.

Differential Cell Count

	Adult (%)	*Child (%)*	*Infant (%)*
Basophils	0.1	0.06	0.07
Eosinophils	3.1	3.6	2.6
Hemocytoblasts	0.1–1.0		
Lymphocytes (all stages)	2.7–24	16	49
Megakaryocytes	0.03–0.5	0.1	0.05
Plasmacytes	0.1–1.5	0.4	0.02
Promyelocytes	0.5–8.0	1.4	0.76
Reticulum cells	0.1–2.0		
Undifferentiated cells	0.0–0.1		
Neutrophils, total	56.5	57.1	32.4
Metamyelocytes	9.6–24.6	23.3	11.3
Neutrophilic	10–32		
Eosinophilic	0.3–3.7		
Basophilic	0–0.3		
Monocytes (all stages)	0–2.7		
Myeloblasts	0.1–5.0	1.2	0.62
Myelocytes	4.2–15	18.4	2.5
Neutrophilic	5.0–20		
Eosinophilic	0.1–3.0		
Basophilic	0–0.5		
Segmented granulocyte	6.0–12.0	12.9	3.6
Neutrophilic	7.0–30		

Contd.

	Adult (%)	*Child (%)*	*Infant (%)*
Eosinophilic	0.2–4.0		
Basophilic	0–0.7		
Band cells	9.5–15.3	0	14.1
Neutrophilic	10–35		
Eosinophilic	0.2–2.0		
Basophilic	0.3		
Erythroid series			
Normoblasts, total	25.6	23.1	8.0
Pronormoblasts	0.2–4.0	0.5	0.1
Basophilic normoblasts	1.5–5.8	1.7	0.34
Polychromatophilic normoblasts	5.0–26.4	18.2	6.9
Orthochromic normoblasts	3.6–21	2.7	0.54
Promegaloblasts	0		
Basophilic megaloblasts	0		
Polychromatic megaloblasts	0		
Orthochromic megaloblasts	0		

M:E ratio: (Myeloid: Erythroid is the ratio of WBCs to nucleated RBCs)

Adult	6:1–2:1
Birth	1.85:1
2 weeks	11:1
1–2 months	5.5:1
1 1–20 years	2.95:1

Usage: Helps to distinguish primary and meta-static tumors. Assists in the identification, classification, and staging of neoplasias. Aids evaluation of the progress and/or response to the treatment of neoplasias. Assists in the definitive diagnosis of blood disorders. Culture of an aspirated sample can aid in the identification of infections such as histoplasmosis or tuberculosis. Histologic examination aids in the diagnosis of carcinoma, granulomas, lymphoma, or myelofibrosis. Iron stain showing decreased hemosiderin levels may indicate iron deficiency and SBB stain differentiates acute granulocytic leukemia from acute lymphocytic leukemia.

Increased eosinophils: Bone marrow carcinoma, eosinophilic leukemia, lymphadenoma, myeloid leukemia and pernicious anemia (relapse).

Increased lymphocytes: Aplastic anemia, hypoplasia of the bone marrow, infectious lymphocytosis or mononucleosis, lymphatic leukemoid reactions, lymphocytic leukemia (B-cell and T- cell), lymphoma, macroglobulinemia, myelofibrosis and viral infections.

Increased megakaryocytes: Acute hemorrhage, ageing, chronic myeloid leukemia, hypersplenism, idiopathic thrombocytopenia, infection, megakaryocytic myelosis, myelofibrosis, pneumonia, polycythemia vera and thrombocytopenia.

Increased plasma cells: Agranulocytosis, amyloidosis, aplastic anemia, carcinomatosis, collagen disease, hepatic cirrhosis, Hodgkin's disease, hypersensitivity reactions, infection, irradiation, macroglobulinemia, malignant tumor, multiple myeloma, rheumatic fever (acute), rheumatoid arthritis, serum sickness, syphilis and ulcerative colitis.

Increased granulocyte: Hypoplasia of the bone marrow, infections, myelocytic leukemia, myelocytic leukemoid reaction and myeloproliferative syndrome.

Increased normoblasts: Anemia (iron deficiency, hemolytic, megaloblastic), blood loss (chronic), erythema, erythroid-type myeloproliferative disorders, hypoplasia of the bone marrow and polycythemia vera.

Increased M:E ratio above 7:1: Decreased hematopoiesis, erythroid hypoplasia, infection, leukemoid reactions, and myeloid leukemia.

Increased diffuse bone marrow hyperplasia: Myeloproliferative syndromes and pancytopenia reactions.

Decreased megakaryocytes: Anemia (aplastic, pernicious), bone marrow hyperplasia (with carcinomatous or leukemic deposits), cirrhosis, irradiation (excessive), and thrombocytopenia purpura. Drugs include benzene, chlorothiazides, and cytotoxic drugs.

Decreased granulocytes: Agranulocylosis, hyperplasia of the bone marrow, and ionizing radiation.

Decreased normoblasts: Anemia (aplastic, hypoplastic), folic acid or vitamin B_{12} deficiency.

Decreased M:E ratio below 2:1: Agranulocylosis, anemia (iron deficiency, normoblastic, pernicious, posthemolytic, posthemorrhagic), erythroid activity (increased), hepatic disease, myeloid formation (decreased), polycythemia vera, sprue, and steatorrhea.

Decreased diffuse bone marrow hypoplasia: Aging, cellular infiltrations, dengue fever, myelofibrosis, myelosclerosis, myelotoxic agents, osteoporosis, rubella, and viral infections.

Description: Bone marrow is the soft, organic, sponge-like material contained in the medullary cavities, long bones, some haversian canals, and within the spaces between trabeculae of cancellous bone. It is composed of red and yellow marrow, with the chief function being production of erythrocytes, leukocytes, and platelets. Only the rusty, red marrow produces blood cells. The yellow marrow is formed of connective tissue and fat cells, which are inactive. During infancy and childhood, bone marrow is primarily red marrow, and in the adult, 50% is red marrow. The bone marrow aspiration procedure obtains a sample of bone marrow by needle. A stained blood smear of the sample is evaluated for bone marrow morphology and examination of blood cell erythropoiesis, cellularity, differential cell count, bone marrow iron stores, and M:E ratios.

Indications for Bone Marrow Aspiration

Absolute Indications

- Megaloblastic macrocytic anemia.
- Aleukemic or subleukemic leukemia.

Diagnostic Importance

- Multiple myeloma
- Aplastic anemia
- Gaucher's disease

Confirmatory Importance

- Leukemias of all types
- Hemolytic anemia
- Idiopathic thrombocytopenic purpura
- Idiopathic granulocytopenia
- Leishmaniasis
- Disseminated lupus erythematosus (LE cells)
- Metastatic disease.

- Myeloproliferative disorders
- Lipid storage
- Sideroblastic anemia
- Iron deficiency anemia
- Lymphoma (staging)

Therapeutic Importance

Bone marrow may be obtained from one person for transplantation into another.

In many cases one may just obtain blood (*blood tap*) or nothing at all (*dry tap*). Under these conditions, a bone marrow biopsy has to be performed. It can be obtained with Jamshidi's needle or with Sacker–Nordins bone biopsy trephine.

Bone biopsy may be needed in

- Malignant lymphoma
- Metastatic carcinoma
- Sarcoidosis
- Tuberculosis
- Brucellosis
- Histoplasmosis
- Storage disorders
- Aplastic anemia
- Myelofibrosis
- Acute leukemias

Contraindications

- Hemophilia.
- Bleeding diathesis.
- Infection at site of puncture.

RETICULOCYTE COUNT

Reticulocytes. These are immature red cells which still contain the remains of ribonuclear protein. Their number in peripheral blood increases following increased erythroid activity in the bone marrow. This may occur when there is reduction in number of red cells in the peripheral blood, e.g. by hemorrhage or abnormal hemolysis. The reticulocyte count is of value in pernicious/megaloblastic anemias, for improvement is indicated by a rise in reticulocytes in the peripheral blood.

Normal Values

Comprises 1–2% of the total RBC count

		SI Units
Adult females	0.5–2.5%	$0.005\text{-}0.025 \times 10^{-3}$
Adults males	0.5–1.5%	$0.005\text{-}0.015 \times 10^{-3}$
Cord blood	3.0–7.0%	$0.030\text{-}0.070 \times 10^{-3}$
Newborn	1.1–4.5%	$0.011\text{-}0.045 \times 10^{-3}$
Neonates	0.1–1.5%	$1.010\text{-}0.015 \times 10^{-3}$
Infants	0.5–3.1%	$0.005\text{-}0.031 \times 10^{-3}$
Children >6 months	0.5–4.0%	$0.005\text{-}0.040 \times 10^{-3}$

Staining

Ribonuclear protein is a living material which requires special staining, using a supravital stain such as brilliant cresyl blue. Methyl alcohol destroys ribonuclear protein; and hence, it cannot be seen in Romanowsky stained pre-paration. In Romanowsky stained films, they appear as larger cells showing polychromasia.

Stain

- 1% Brilliant cresyl blue
- Brilliant cresyl blue 1 g
- Sodium chloride 0.7 g
- Sodium citrate 0.6 g
- Distilled water 100 mL

Store in dark bottle under refrigeration, filter before use (new methylene blue can also be used instead of BCB).

Method

- Place I volume of filtered stain in small test tube.
- Place I volume of capillary or venous blood and mix.
- Incubate at room temperature or at 37°C for 10–30 minutes.
- Remix the tube contents and spread 1 drop of the stained blood on a slide making a thin film.

When dry, examine with the oil immersion lens. Count systematically at least 500 cells, including in this the number of reticulocytes. Reticulocytes appear larger than mature cells and contain irregular dark purple granules or fine threads of ribonuclear material, calculate the percentage of reticulocytes.

Interpretation: Reticulocyte counts are low in ineffective erythropoiesis, e.g. myelosclerosis, aplastic anemia, megaloblastic anemia, thalassemia, erythroleukemia and sideroblastic anemia. Reticulocytosis occurs after blood loss or effective therapy for certain kinds of anemia, e.g., therapy of iron deficiency or megaloblastic macrocytic anemia. Reticulocytosis also occurs in hemolytic anemias.

RED CELL FRAGILITY TEST

Screening test: One needs 0.45% sodium chloride solution and hemocytometer for counting red cells. Blood is drawn to the 0.5 mark in 2 red cell pipettes. The first is diluted to the 101 mark with Hayem's (RBC diluting fluid) and the second with 0.45% sodium chloride solution. Both pipettes are shaken for 2 minutes and counts made from both pipettes, the percentage of cells hemolyzed in the 0.45% saline solution is thus determined. Less than 30% of normal erythrocytes are hemolyzed by this technique. An abnormal increase in red cell fragility, as in congenital hemolytic icterus will cause hemolysis of more than 70% of the cells.

Quantitative Test

Principle: Tubes containing solution of varying concentration of saline buffered to pH 7.4 are used. Heparinised or defibrinated blood is added to each tube in a proportion of 1–100 and the degree of hemolysis in each is noted using a photoelectric colorimeter. The result may be reported as a graph or stating the concentration at which hemolysis begins and that at which it is complete.

Reagents

A stock solution of buffered sodium chloride (AR) osmotically equivalent to 10% NaCl, is made up as follows. NaCl 180 g, Na_2HPO_4 27.31 g and $NaH_2PO_42H_2O$ 4.86 g are dissolved in distilled water and the final volume adjusted to 2 liters. This solution will keep for months in a well-stoppered bottle. In preparing solutions for use it is convenient to make first a 1% solution from the 10% stock solution by dilution with distilled water. Dilutions equivalent to 0.85, 0.75, 0.65, 0.60, 0.55, 0.45, 0.40, 0.35, 0.30, 0.20 and 0.10% NaCl are convenient test concentrations. Intermediate concentrations such as 0.475% and 0.525% are useful in critical work.

Distilled water drop	18	17	16	15	14	13	12	11	10	9	8	7
1% NaCl	9	10	11	12	13	14	15	16	17	17	17	18
NaCl%	0.28	0.32	0.36	0.40	0.44	0.48	0.52	0.56	0.60	0.64	0.68	0.72

If the test is performed very occasionally, smaller volumes of the solutions may be made up as given at the bottom of this page.

Method

Use: Heparinized or defibrinated blood, oxalated and citrated blood may change the tonicity which is not desirable.

Add 0.05 mL of blood to each tube containing 5 mL of the different concentrations of saline. Mix well and let the tubes stand at room temperature for 30 minutes. Remix and centrifuge for 5 minutes at 2000 rpm and measure the amount of hemolysis in each tube in a photoelectric colorimeter with green filter. The supernatant from 0.85% NaCl is used as the blank because there is no hemolysis in this concentration (normal) of saline. The supernatant from the 0.1% NaCl is used to estimate 100% lysis (the supernatant can easily be decanted into the cuvette of the colorimeter). The depth of color should be such that the reading on the colorimeter scale for complete lysis does not exceed 50 (optical density 0.5). If necessary, the supernatant may be diluted with an equal volume of 0.1% NaCl or the initial proportion of blood may be 1:200 instead of 1:100. With a good colorimeter, as little as 1% hemolysis may be detected.

The blood added should be exactly 0.05 mL. It can be done by using capillary automatic pipettes. Alternatively straight glass pipettes graduated till 0.05 mL may be used. Less time consuming and far less accurate method is to add one drop of blood to each tube.

Factors Affecting Osmotic Fragility Tests

In carrying out osmotic fragility tests by any method three variables capable of markedly affecting the results must be controlled, quite apart from the accuracy with which the saline solutions have been made up. These are; (i) the relative volumes of blood and saline, (ii) the final pH of the blood-saline suspension, and (iii) the temperature at which the tests are carried out.

Interpretation

Spherocytes, being already round are unable to swell very much and therefore, rupture even when a small amount of water has entered the cell. Hemolysis may thus commence even at 0.75% and may be complete at 0.4% (a feature of spherocytosis). On the other hand, target cells seen in thalassemias and iron deficiency anemia cells can swell a great deal before they rupture because they are relatively flat. Fragility is, therefore, said to be decreased.

Clinical Implications

a. Increased fragility (>0.5%) occurs in
   1. Hereditary spherocytosis
   2. Hemolytic jaundice
   3. Autoimmune anemia (ABO and Rh) incompatibility
   4. Chemical poisons
   5. Burns.

b. Decreased fragility (<0.3%) occurs in
   1. Obstructive jaundice
   2. Thalassemia
   3. Sickle cell anemia
   4. Iron-deficiency anemia
   5. Polycythemia vera
   6. Liver disease
   7. Splenectomy (following)

Decreased fragility indicates that red cells are excessively flat. Occurs in iron deficiency anemia, thalassemia, and sickle cell disease.

QUALITATIVE ASSESSMENT OF G6PD DEFICIENCY

Methemoglobin Reduction Test

Reagents

1. Sodium nitrite 1.25 g in 100 mL distilled water.
2. Glucose 5 g in 100 mL distilled water.
3. Methylene blue 150 mg in 100 mL distilled water.

Method

Withdraw 6 mL of blood and add to 1.2 mL of ACD solution.
Label three test tubes as A, B and C, add as follows:

a. To tube A, add:
   - 0.1 mL sodium nitrite solution
   - 0.1 mL glucose solution
   - 0.1 mL methylene blue solution
   - 2 mL blood.

b. To tube B, add:
   - 0.1 mL sodium nitrite solution
   - 0.1 mL glucose solution
   - 2 mL blood.

c. To tube C, add 2 mL of blood only.

Mix well and keep the tubes A, B and C at 37°C for 3 hours. Mix again and aerate at 1, 2, and 3 hours (hourly intervals).

Take three test tubes each containing 10 mL of distilled water.
To one, add 0.1 mL of mixture of A.
To second, add 0.1 mL of mixture of B.
To third, add 0.1 mL of contents from C.
Wait for 10 minutes.
Test tube with distilled water and contents from C should always be red.
Test tube with distilled water and solution from B should always be brown.

Interpretation

Test tube with distilled water and solution from A.

- If this is red—there is no G6PD deficiency
- If brown like B—full expression of deficiency of G6PD
- If between red and brown—intermittent expression of G6PD deficiency.

Aging red cells are especially susceptible to oxidative challenge by drugs, systemic infection, metabolic acidosis and other stress. Oxidative stress induces rapid intravascular destruction of susceptible cells, leading to hemoglobinemia, hemoglobinuria and a sudden drop of hematocrit.

Young cells have higher G6PD content than the older ones, regardless of the genetic variant that is present. If the enzyme has defective activity, older cells are preferentially destroyed during a mild to moderate hemolytic phase. Reticulocytes released to replace lost cells have high enzyme levels. False-negative test results often occur if blood is examined just after a hemolytic episode, because the nonhemolyzed remaining cells are, by definition, those with adequate enzyme levels. Newly generated reticulocytes have still higher levels, and this can affect the results for 3–10 days after the episode.

Drugs that hemolyze G6PD deficient cells are those that either act as direct oxidants themselves or produce peroxide activity. Primaquine, an antimalarial drug is notable in this respect. Many sulpha drugs, quinine derivatives, nitrofurans and antipyretic–analgestic drugs can induce hemolysis in G6PD deficient patients. Susceptibility seems to vary among different individuals. The presence of coexisting fever, metabolic disease, or hepatic or renal failure increases likelihood that symptoms will emerge.

QUANTITATIVE ESTIMATION OF G6PD

Courtesy: *Tulip Group of Companies*

Summary

Glucose-6-phosphate-dehydrogenase (G6PDH) deficiency is one of the most common human enzyme deficiencies in the world. During G6PD deficiency, the red cells are unable to regenerate reduced nicotinamide adenine dinucleotide phosphate (NADPH), a reaction that is normally catalyzed by the G6PD enzyme. Since the X chromosome carries the gene for G6PD enzyme, this deficiency mostly affects the males.

The two major conditions associated with G6PD deficiency are hemolytic anemia and neonatal jaundice, which may result in neurological complications and death. Screening and detection of G6PD deficiency helps in reducing such episodes, through appropriate selection of treatment, patient counseling and abstinence from disease precipitating drugs such as antimalarials and other agents.

Principle

G6PDH in the RBCs is released by a lysing agent present in the reagent. The G6PDH released catalyzes the oxidation of glucose 6 phosphate with the reduction of NADP to NADPH. The rate of reduction of NADP to NADPH is measured as an increase in absorbance, which is proportional to the G6PDH activity in the sample.

$$\text{G-6-P} + \text{NADP} \xrightarrow{\text{G-6-PDH}} \text{Gluconate-6-P} + \text{NADPH} + \text{H}$$

Normal Reference Values

G6PDH Activity (U/g Hb.): 4.6–13.5 at 30°C/6.4–18.7 at 37°C
(U/10^{12} RBC's): 146–376 at 30°C/202–522 at 37°C

It is recommended that each laboratory establishes its own normal range representing its patient population.

Contents	*5 × 1 test*	*5 × 5 tests*
L1 : G6PDH Reagent	5 × 1 mL	5 × 5.5 mL
L2 : Starter Reagent	10 mL	50 mL

Storage/Stability

Contents are stable at 2–8°C till the expiry date mentioned on the labels.

Reagent Preparation

Reconstitute G6PDH reagent (L1) with D.W. as per the volume mentioned on the label. This working reagent is stable for 6 hours at RT and at least 3 days when stored at 2–8°C.

The Starter Reagent (L2) is ready to use.

Sample Material

Fresh whole blood sample collected in EDTA, Heparin or ACD. Red Cell G6PDH in whole blood is reported to be stable for 7 days at 2–8°C, but is unstable in hemolysates. Freezing is not recommended.

Procedure

Wavelength/filter : 340 nm
Temperature: 30°C/37°C
Light path: 1 cm

Addition Sequence	*S (mL)*
G6PD working reagent (L1)	1.0
Whole blood	0.01
Mix well and incubate for 5–10 min at RT and add substrate reagent	2.0

Mix well and incubate for 5 min. at 30°C/ 37°C and read the initial absorbance A0 and repeat the absorbance reading after every 1, 2 and 3 minutes. Calculate the mean absorbance change per minute (DA/min).

If the G6PDH activity is very low, the absorbance change per minute will also be very low. In such cases read the initial absorbance A1 and read another absorbance A2 exactly 5 min. later. Calculate the mean absorbance change per minute (ΔA /min.)

$$A/\text{min} = \frac{A2 - A1}{5}$$

Calculations

$$\text{G6PDH Activity (U/}10^{12}\text{ RBC)} = \Delta A \times \frac{47780}{\text{RBC count (/mm}^3\text{)}}$$

$$\text{G6PDH Activity (U/gHb)} = \Delta A \times \frac{4778}{\text{Hb (g/dL)}}$$

Temperature Conversion Factors			
Assay temperature	*Desired reporting 25°C*	*Temperature*	
		30°C	*37°C*
25°C	1.00	1.32	1.82
30°C	0.76	1.00	1.39
37°C	0.55	0.72	1.00

Notes

Since the activity of G6PDH is reported in Hb, concentration or RBC count the same should be determined before performing the assay. RBCs are well preserved when collected in ACD and such samples give an accurate count, for samples collected in heparin counts become unreliable after 2 days and in such cases results are best reported in Hb concentration.

Copper and sulphate ions inhibit the G6PDH activity; hence use of good quality deionized or distilled water for reconstitution of L1 and properly cleaned glassware is essential.

Young red cells have a higher G6PD content then the older ones, regardless of the genetic variant that is present. If the enzymes have defective activity, older cells are preferentially destroyed during mild to moderate hemolytic phase. Since reticulocytes released to replace lost cells have high enzyme levels, falsely elevated results may occur if blood is tested immediately after a hemolytic episode.

Normally the activity contributed by WBC, platelets or serum is very small. In cases of severe anemia, leukocytosis, or very low G6PDH levels, the use of a sample after removing the buffy coat is recommended.

EXAMINATION OF FETAL HEMOGLOBIN

Qualitative Method

Peripheral blood film staining method. (Acid elution technique).

Always use freshly prepared smears

- Fix in 80% ethanol for 10 minutes.
- Take 37.7 mL of citric acid solution prepared (21 g of citric acid by dissolving, in 100 cc of distilled water) in a jar kept at 37°C.
- To this, add 12.2 mL of $Na_2H\ PO_4$ solution (prepared by dissolving 57.6 g of $Na_2H\ PO_4.12H_2O$ (in 1000 cc of distilled water).
- In this mixture keep the slide for 30 minutes.
- Wash with distilled water.
- Stain with 1% eosin solution for 5 minutes.

Interpretation

Normal red cells will appear as ghost cells. Fetal hemoglobin containing cells will appear as bright red cells.

Quantitative Method

STEP A

Preparation of hemolysate (Fig. 24.13)

1. Take 8 cc of EDTA blood, centrifuge it for 15 minutes, remove the plasma.

2. To the RBCs sediment, add normal saline and fill the centrifuge tube to 4/5th of it. Mix thoroughly and centrifuge for 20 minutes. Remove the saline and repeat the said process at least twice more.
3. To the packed RBCs, add equal quantity of distilled water and half the quantity of toluene. Mix them thoroughly and keep in the deep freezer for one hour.
4. Remove from the freezer chest. Thaw it and centrifuge it for 30 minutes. Now there will be 3 zones in the tube.
5. Pass a pipette to the hemolysate zone through the side of the test tube without disturbing the upper two zones, suck up the hemolysate.
6. Check the hemoglobin of the hemolysate and adjust to 8–10 g%. This hemolysate can be stored at –20°C for 6 months and can be used for quantitative (alkali denaturation method) estimation of fetal hemoglobin or for hemoglobin electrophoresis.

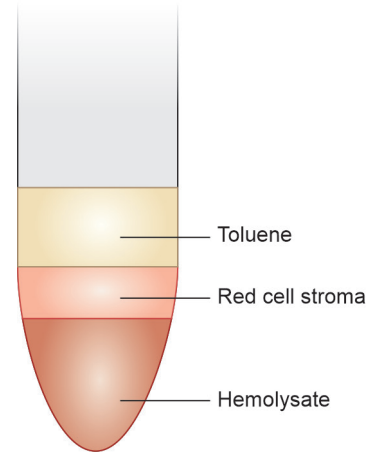


Fig. 24.13: Preparation of hemolysate

STEP B

Quantitative alkali denaturation method for estimation of fetal hemoglobin

1. Take 24.5 mL of Drabkin's solution in a test tube, add 0.5 mL of hemolysate. Cyanmet-hemoglobin (HiCN) is formed. Mix well.
2. Transfer 2.8 mL of HiCN solution in a tube and keep at 20°C. To this, add 0.2 mL of 1.2 N-NaOH solution. Mix rapidly—incubate for 2 minutes at 20°C. Add 2 cc of saturated solution of ammonium sulphate. Mix again and let stand for 5–10 minutes. Filter through Whatman filter paper number 3.
3. Prepare the total hemoglobin by adding 0.4 mL of the original HiCN solution to 6.5 mL of distilled water.
4. Using Drabkin's solution as blank at 540 nm, read the total hemoglobin and filtrate.
5. The optical density should fall between 0.05 and 0.5. If it is beyond 0.5, dilute the hemolysate with distilled water and repeat the said procedure.
6. Calculate as
   Hemoglobin F (percentage of HbF)

$$\frac{\text{OD of filtrate}}{\text{OD of total hemoglobin}} \times \frac{100}{10}$$

HbF and HbS concentration as percentage of total Hb concentration in various disorders is given below:

Disorder	*HbF%*	*HbS%*
Normal	<1% (in adults)	Not present
Sickle cell trait (AS)	Normal	30–40%
Sickle cell anemia (SS)	1–20%	75–95%
HbS β thalassemia		
[$S\beta^+$ (some β chains present)]	2–10%	60–85%
[$S\beta^0$ (no β chains present)]	5–30%	76–90%
HbS-C (SC)	1–5%	50-55%
HbS-D (SD)	1–5%	95% (S+D)
β Thalassemia major	10–98%	—
α Thalassemia	Reduced	—

Normal Values

Adults	0–2% of total hemoglobin
Children	
Newborn	<60–90% of total hemoglobin
1–5 months	<75% of total hemoglobin
6–12 months	<5% of total hemoglobin
1–20 years	<2% of total hemoglobin

TESTS FOR SICKLING

Sickle cells are abnormal forms resulting from the presence of abnormal hemoglobin, which in the deoxidized state undergoes a hydrophobic-bond dependent polymerization. Rigid threads or rods of S hemoglobin result, with consequent production of the rigid sickle cells. Sickling occurs in 10% of blacks, but only a few of these have anemia.

Methods

Moist Cover Slip Preparation

Cover a small drop of fresh blood on a slide with a coverslip, seal with petrolatum, paraffin or nail polish, keep at warm room temperature and examine for sickling every 12 hours up to 72 hours. To find percentage of circulating sickling forms, draw 4 mL of blood into a syringe containing 1 mL of 10% formalin, examine wet preparation.

Daland and Da Silva Method

Acceleration of removal of oxygen from hemoglobin by use of reducing substances.

Reagents

Sodium bisulphite or ascorbic acid, 2 gm, in 100 mL water (to be made fresh each time).

Method

Use capillary blood, oxalated or defibrinated. Mix 2 drops of either reagent with one drop of blood on a glass slide. Cover, and seal coverslip with petrolatum or nail polish. If no sickling appears in 4 hours, the test is negative.

Positive test: 10% or more red cells show sickled forms.

Dithionite Tube Test

The red cells are lysed, hemoglobin deoxygenated, and the β-globin chains are displaced to provide the molecular steric fit characteristic of sickling of S and no S hemoglobins. The test solution becomes turbid if sickling hemoglobins are present.

Reagents

Stock solution

KH_2PO_4	160.48 g
K_2HPO_4	281.18 g

Distilled water to 1 liter.

Working Solution

Stock buffer	800 mL
$Na_2S_2O_4$	60 g
Saponin (5%) solution	20 mL
Distilled water to	1000 mL

Method

Add 20 µL (0.02 mL) of well mixed whole blood (EDTA anticoagulated) to 2 mL dithionite working solution in a 12 × 75 mm test tube. Mix by inverting and allow to remain at room temperature for 5–6 minutes. Examine for opacity by holding the tube 2.5–3 cm from a sheet of black newsprint.

Interpretation

Turbidity (newsprint not visible) indicates the presence of sickling hemoglobin. Clear solution indicates a negative test. A positive test (turbidity) indicates the presence of any of the hemoglobins S (SS or AS), C (Harlem), C (Georgetown), Barts and perhaps Alexandra. All positive tests should be followed by hemoglobin electrophoresis. Hemoglobin S can be separated from other hemoglobins by the urea–dithionite test.

Urea–Dithionite Test

Reagents

To 50 mL of working solution (above: from previous test), add 6 g of urea. Mix until dissolved.

Method

Add 20 µL (0.02 mL) whole blood to 2 mL of urea–dithionite solution in a 12 × 75 mm test tube. Mix by inverting tube and allow to remain at room temperature for 5 minutes. Read with tube 2.5–3 cm away from newsprint background.

Interpretation

Turbidity produced by the sickled liquid crystal system of hemoglobin S in dithionite is dispersed in the urea–dithionite by the urea, which breaks the hydrophobic bonds essential to sickling.

Hemoglobin	*Dithionite*	*Urea–dithionite*
SS, AS and C (Harlem)	Turbid	Clear
Nonsickling C (Georgetown), Barts, Alexandra	Turbid	Turbid

The presence of HbA guards, to a modest extent, against tactoid (polymerization) formation, HbF exerts a very strong protective effect. It is probable that the cells of patients with sickle cell disease undergo intravascular sickling and unsickling many times as they circulate. Repeated shape changes, however, stress the cell membrane and cause the loss of small membrane fragments. This reduces the ratio of surface to volume, making the cell less flexible and less responsive to physical changes. Some cells gradually loose their mechanical and osmotic resistance and undergo intravascular dissolution, whereas others are removed early by reticuloendothelial system. Red cells in HbS disease experience a chronically shortened life span.

The likelihood of sickling increases with low oxygen tensions, lowered pH and increased body temperature. HbS protects against *Plasmodium falciparum* infection. Sickle cell anemia rarely becomes clinically apparent before 6 months of age because protective amounts of HbF remain in cell; for the same reason, it is difficult to screen newborns for HbS disease.

LABORATORY DIAGNOSIS OF LEUKEMIAS

Cytochemical Methods for Staining Leukocytes

Neutrophil Alkaline Phosphatase (Kaplow's Method)

Principle: The enzyme, located in the neutrophil specific granules, is exposed to the substrate (a naphthol phosphate) in the presence of a diazonium salt (fast blue or fast violet) at an alkaline pH, 9.5. The substrate is hydrolyzed by the enzyme, releasing a phosphate and an aryl naphthol amide. The latter is immediately coupled to the diazonium salt, forming an insoluble azo dye.

Reagents

- *Fixative:* 10% formalin in absolute methanol. To 10 mL 37% formal dehyde, add 90 mL absolute methanol. Store at 10–20°C.
- *Buffer stock:* 0.2 M propanediol. Dissolve 21 g of 2-amino-2 methyl-1, 3-propanediol in distilled water and dilute to 1000 mL. Store at 4°C.
- *Working:* 0.05 M propanediol pH 9.4–9.6. Add 70 mL 0.1 N HCI to 250 mL of stock buffer and dilute to 1000 mL with distilled water. Store at 4°C.
- *Substrate mixture:* Dissolve 5 mg of naphthol AS-BI phosphate or naphthol AS-MX phosphate or naphthol AS phosphate in 0.2–0.3 mL dimethyl formamide in a dry flask and add 60 mL of 0.05 M propanediol buffer and 40 mg of fast blue salt RR, BB, or BBN (or fast red violet LB). Shake well, filter into a Coplin jar and use immediately.
- *Counterstain:* Mayer's hematoxylin. Add 1 g. hematoxylin to 500 mL distilled water. Heat just to boiling and add another 500 mL distilled water. Add 0.2 g sodium iodate and 50 g of aluminum potassium sulfate. Shake well, filter and store in brown bottle at room temperature.

Procedure

- Use freshly made blood films. If venous blood is used, heparin should be the anticoagulant, as the enzyme activity diminishes rapidly in EDTA.
- Fix air dried blood films in 10% formal methanol for exactly 30 seconds at 0 to –10°C.
- Wash in gently running tap water for 30–60 seconds.
- Air dry slides, then place them in substrate mixture for exactly 10 minutes. Wash in gently running tap water again for 30–60 seconds.
- Counterstain for 6–8 minutes in filtered Mayer's hematoxylin.

FAB Classification of lymphoblastic leukemias

Observation	*L1*	*L2*	*L3*
Consistency of appearance	Homogeneous	Heterogeneous	Homogeneous
Cell size	Uniformly small	Large, but variable	Uniformly large
Nuclear shape	Regular, little clefting	Irregular, clefted, indented	Regular, rounded
Nucleoli	None or inconspicuous	One or more large	One or more, prominent
Amount of cytoplasm	Scant	Variable, often abundant	Abundant
Other findings	T d T* usually	T d T usually Myeloperoxidase negative	May have B cell markings

*(T d T—Terminal deoxynucleotidyl transferase)

Wash in running tap water for 2 minutes. Air dry. Positive controls are run with each batch of slides. Women in last trimester of pregnancy are good controls, because their scores are high normal or somewhat increased.

Scoring Procedure

Examine 100 mature neutrophils in the thin part of the film, where red cells barely touch each other and score each as follows:

Unstained cells	0
Cells stained faintly diffusely, or a few discrete granules	1
Cells with moderate number of granules	2
Cells with granules filling the cell	3
Cells staining deeply, almost obscuring the nucleus	4

Adding the scores for 100 cells can give a possible range of 0–400. The normal range with this method is 20–100.

The NAP scores are raised in:

- Bacterial infections
- Myocardial infarction
- Trauma
- Diabetic acidosis
- Polycythemia vera
- Myelosclerosis
- Following corticosteroid therapy
- During pregnancy
- Use of oral contraceptives.

The NAP scores are lowered in:

- Chronic myeloid leukemia
- Paroxysmal nocturnal hemoglobinuria } always
- Idiopathic thrombocytopenic purpura
- Infectious mononucleosis
- Pernicious anemia relapse
- Collagen disorders and refractory anemias } sometimes
- Hypophosphatemia

Peroxidase (Myeloperoxidase, Kaplow's Method)

Principle: In the presence of hydrogen peroxide, peroxidase in leukocyte granules oxidises benzidine from a colorless form to blue or brown derivative which is localized at the site of the enzyme.

Reagents

Fixative: Mix 10 mL of 37% formal dehyde with 90 mL of absolute ethanol:

Incubation mixture	
Ethanol 30% (v/v) in water Benzidine dihydrochloride Zn SO_4. $7H_2O$, 0.132M 3.8% w/v)	100 mL 0.3 g 1.0 mL
Sodium acetate	
($Na_2C_2H_3O_2$. $3H_2O$) 3% hydrogen peroxide Sodium hydroxide, 1.0 N Safranin O	1.0 g 0.7 mL 1.5 mL 0.2 g

Reagents are mixed in the stated order. A precipitate forms after adding zinc sulfate but dissolves after other reagents are added. The pH is not critical between 5.8 and 6.5. The mixture is filtered and may be kept in a closed container and reused for a period of several months.

Procedure

Freshly made films or imprints are used. Peroxidase is unstable in the light, but unfixed films are satisfactory for as long as 3 weeks if kept in the dark. Heparin/oxalate/EDTA can be used as an anticoagulant.

Place slides in fixative for 60 seconds at room temperature. Wash in gently running tap water.

Place slides in incubation mixture for 30 seconds at room temperature. Wash in gently running tap water for 30–60 seconds.

Allow slides to dry, and examine under the microscope.

The slides may be counterstained with Wright's stain or with 1% aqueous cresyl violet if greater nuclear detail is wanted.

Interpretation

Peroxidase activity is indicated by blue granules in the cytoplasm. The nucleus and background cytoplasm stain red.

In neutrophilic series peroxidase becomes positive in late myeloblasts and on till mature neutrophil. In eosinophils specific granules contain peroxidase. Basophils, lymphocytes and erythroid cells do not stain. Monocytes stain less intensely than do neutrophils, and the granules are smaller.

Peroxidase reaction is used in differentiating acute myeloblastic leukemia (+ve) from acute lymphoblastic leukemia (–ve). It parallels Sudan Black B reaction; Auer rods are positive with both.

Peroxidase activity may be absent in some toxic neutrophils in infection.

Periodic Acid–Schiff (PAS) Reaction

Principle: Periodic acid (HIO_4) is an oxidizing agent that converts hydroxy groups on adjacent carbon atoms to aldehydes. The resulting dialdehydes are combined with Schiff's reagent to give a red-colored product. A positive reaction is, therefore, seen with polysaccharides, mucopolysaccharides, and glycoproteins.

Reagents

- Fixative: Mix 10 mL of 37% formaldehyde with 90 mL of absolute ethanol.
- Periodic acid, 5 g, is dissolved in 500 mL of distilled water. Stored in dark bottle and is good for 3 months.
- Schiff's reagent: Dissolve 5 g of basic fuchsin in 500 mL of hot distilled water and filter after it has cooled. Saturate with sulfur dioxide gas by bubbling for 1 hour. Extract the solution with 2 g. of activated charcoal for a few seconds in a hood and immediately filter through Whatman No. 1 filter paper into a dark bottle. The solution is kept for 2–3 months.
- Harris hematoxylin.

Procedure

- Place air-dried blood and marrow films or imprints in fixative for 10 minutes. Wash briefly with tap water.

- Control slides are exposed to digestion with saliva (diastase) for 30 minutes. Place slides in periodic acid for 10 minutes. Wash briefly with tap water and blot dry.
- Immerse slides in Schiff's reagent for 30 minutes.
- Rinse slides in several changes of sulfur dioxide water for 20–30 minutes.
- Wash for 5–10 minutes in tap water and counterstain with Harris hematoxylin for 10 minutes.

Interpretation

In blood cells a positive PAS reaction usually indicates presence of glycogen. This is shown by digestion with diastase and consequent loss of staining.

Neutrophils react at all stages of development, the most strongly in the mature stage (similarly for eosinophils). The glycogen is not in the granules but in the background cytoplasm. Myeloblasts contain a few small PAS-positive granules. Monocytes have a faint staining reaction in the form of fine granules. Lymphocytes may contain a few small or large granules. Normoblasts are normally PAS-negative.

In erythroleukemia and in thalassemia some of the erythroid precursors are PAS positive. In acute lymphoblastic leukemias, the lymphoblasts often contain large coarse clumps of PAS-positive material (block positivity).

Sudan Black B Stain (Sheehan and Storey)

Principle: Sudan Black B stains phospholipids and other lipids. It appears to stain both azurophilic and specific granules in neutrophils, whereas the peroxidase is found only in azurophilic granules. In early forms, late myeloblasts and early promyelocytes, the Sudan Black B reaction is therefore, parallel to the peroxidase in its utility in separating acute lymphoblastic from acute myeloblastic leukemia.

Reagents

- *Stock solution of stain:* Dissolve 0.3 g of Sudan Black B powder in 100 mL ethyl alcohol.
- *Buffer solution:* Dissolve 16 g crystalline phenol in 30 mL ethyl alcohol. Add this to a solution of 0.3 g hydrated disodium hydrogen phosphate (Na_2HPO_4. $12H_2O$) dissolved in 100 mL distilled water.
- *Working stain solution:* Add 40 mL buffer solution to 60 mL stock stain solution. Filter using suction. This is stable for approximately 2 months.

Procedure

- Fix air dried films in formalin vapor for 10 minutes. Slides need not be freshly made.
- Wash slides in running tap water for 10 minutes.
- Place slides in working stain solution (in Coplin jar) for 60 minutes.
- Wash slides with 70% ethyl alcohol for 2–3 minutes to remove excess dye.
- Wash slides in tap water for 2 minutes.
- Allow slides to dry. Counterstain slides with Wright's stain or hematoxylin.

Interpretation

Cytoplasmic granules stain faintly in neutrophil precursors and strongly in mature neutrophils with a brown black color. Eosinophilic granules are brown but often show a central pallor. Monocytes have scattered fine brown-black granules. Lymphocytes and lymphoblasts are negative, but at least some myeloblasts contain Sudan Black positive granules.

The peroxidase and Sudan Black B reactions show roughly similar patterns in the various cell types. These techniques are most useful in distinguishing myeloblasts from lymphoblasts when large numbers of primitive blast forms are present in acute leukemias.

Nonspecific Esterase (Yam *et al.*)

Alpha-naphthol acetate esterase.

Reagents

- *Fixative:* Buffered formalin and acetone.
  - Formaldehyde, 37%; 25 mL, Na_2 HPO_4, 20 mg, KH_2PO_4 100 mg; distilled water, 30 mL; acetone 45 mL.
  - Buffer Sorensen's phosphate buffer (M/15. pH = 7.6)
- *Incubation mixture:* Add in the following manner
  - Buffer 44.5 mL.
  - Hexazotized parasanilin 3.0 mL
  - Alpha-naphthol acetate 50 g dissolved in 2.5 mL ethylene glycol monomethyl ether
  - Filter mixture through Seitz filter
  - Harris hematoxylin.

Procedure

- Place air dried blood or marrow films in fixative for 30 seconds at 4°C. Wash in running tap water.
- Place slides in incubation mixture for 60 minutes. Wash in running tap water.
- Counterstain with Harris hematoxylin for 10 minutes.

Interpretation

Alpha-naphthol acetate esterase activity is found in monocytes but not in neutrophils or neutrophil precursors, other granulocytes or lymphocytes. It may be found, however, in activated or atypical lymphocytes, in lymphocytes in imprints of active lymphoid tissue, and probably in the poorly differentiated lymphocytes of some lymphomas.

QUALITY CONTROL IN HEMATOLOGY

Quality control in medical laboratories encompasses a set of procedures which ensure that reliable and timely test results are received by the users of laboratory service. Reliability implies both precision and accuracy.

There are four components of quality assurance program:

- Internal quality control (IQC)
- External quality assurance (EQA)
- Standardization
- Proficiency surveillance.

INTERNAL QUALITY CONTROL

Since now most of the labs are dependent on automated machine, it has become extremely important to maintain good internal quality control, which is done by:

Testing Control Sample

The best known method is testing a control sample along side the routine specimen in each batch of test. Control material is either obtained commercially or prepared individually, but its stability and homogeneity should be ensured.

Control Chart (Levy–Jennings or L–J Chart)

In this process when a batch of samples is dispensed (after being run along a control sample), the mean and standard deviation of each diameter is obtained and linear graphs are ruled, showing the +2 standard deviation (SD) limits. Statistically, not more than 1 in 20 samples should fall outside these limits if the system is in control.

Cusum Analysis

Cumulative sum (cusum) charting was introduced in 1960s. Deviation from the largest is plotted in a cumulative manner so that each point represents the sum of all the deviations to date from the mean or target value. This method of plotting exaggerates trends in data. And makes shifts of the mean much more obvious than by other plots. The rules for using the cusum system for quality control are less well defined than for the L–J system.

Duplicate Tests

Well known method for checking precision in clinical analysis is duplicate testing. In this process a few of the specimens that were measured in an earlier batch, are rechecked with the next batch control.

Inbuilt Quality Control

This includes:

- These of cumulative reports of a single patient
- Clinical correation: If a physician cannot interpret a report on clinical grounds, a repeat test with a fresh specimen is indicated.
- Red cell indices: If reports are giving erroneous rise or fall in the red cells indices, this usually points to an error in analysis.
- Blood film examination ultimately helps in double checking the analysis done by the instrument.

EXTERNAL QUALITY ASSESSMENT

The college of American Pathologist first introduced "proficiency testing" survey program in 1960. In the late 60s the British Committee for Standards in Hematology, finally developed the National External Quality Assessment Scheme (NEQAS) for Hematology. Such methods are used by various laboratories all over the world to keep up with international standards.

STANDARDIZATION

Modern diagnostic systems depends on a calibration procedure for accurate performance. Calibrators or testing standards are commercially prepared products, made by a direct comparison with a primary international standard. They are used for accuracy and inter-laboratory harmonization of test results. The calibrator has an assigned value as close to the true value as can be established.

World Health Organization (WHO) provides a wide range of biologically important international reference standard material. Some examples of these which are available for use in hematology are:

a. Hemoglobin preparation
b. Hemoglogin A2 and F
c. Thromboplastin
d. Blood type sera
e. Various coagulation factors.

PROFICIENCY SURVEILLANCE

This is concerned with the pre-analytical parts of the process that require control, if tests are to be reliable and effective. This involves following a standard guideline at various steps of a laboratory analysis.

The steps are:

1. Standard of blood collection tube
2. Phlebotomy technique
3. Identification of sample with special reference to hazardous specimens
4. Maintenance of transportation standards
5. Data processing of results
6. Establishing normal reference values, assessment of the significance of results and taking decisions for further tests.

Technical proficiency has always been the corner stone of the laboratory, but in recent years with the advent of sophisticated instruments and automation, quality control has assumed an even more important role in good laboratory practice. It is the duty of the laboratory staff to ensure that the tests which are carried out are appropriate and to provide reliable analytical results.

PLATELETS, COAGULATION AND BLEEDING DISORDERS: LABORATORY INVESTIGATIONS

Platelet count—Dealt with in depth elsewhere.

CAPILLARY FRAGILITY TEST OF HESS

Rumpel–Leede Sign, Tourniquet Test

1. Inflate sphygmomanometer cuff around arm at 80 mm of Hg pressure for 5 minutes.
2. Look for petechiae in an area 5 cm in diameter just below the elbow.
3. Under normal circumstances the number of petechiae should be less than 5, more than 5 indicate a positive test.

A positive test may be found in reduced capillary resistance (or increased capillary fragility) as in nonthrombocytopenic purpura and scurvy. It may also be positive in thrombocytopenia when the platelet count is below approximately 70,000/mm^3 of blood.

Clinical Implications

1. Increased petechiae formation occurs most commonly in thrombocytopenia and less commonly in: (i) thrombasthenia, (ii) vascular purpura, (iii) senile purpura, and (iv) scurvy.
2. The number and size of petechiae are roughly proportional to the bleeding tendency and possibly to the degree of thrombocytopenia. However, the test can be positive because of capillary fragility in the presence of normal platelet count.
3. Results will be normal in coagulation disorders and vascular disorders.

LABORATORY DIAGNOSIS OF VASCULAR BLEEDING DISORDERS

Hess's test is positive in these cases

Causes and classification

1. Hereditary
   - Hereditary hemorrhagic telangiectasia

2. Acquired
   - Simple easy bruising
   - Senile purpura
   - Purpura of infections
   - Henoch–Schönlein syndrome
   - Scurvy
   - Steroid purpura.

Interfering Factors

1. *Menstruation:* Capillary fragility is normally increased before menstruation.
2. *Infectious disease:* Capillary fragility is increased in measles and influenza.
3. *Age:* Women over 40 with decreasing estrogen levels may have a positive test that is not indicative of a coagulation disorder.
4. *Readministration:* Repetition of test on same arm within 1 week of the first test may lead to error.
5. *Variation:* Results may vary because of differences in texture, thickness, and temperature of the skin.

BLEEDING TIME

The duration of bleeding from a standard puncture wound of the skin is a measure of the function of platelets as well as the integrity of the vessel wall.

Duke's Method

Requirements

1. Stop watch
2. Lancet
3. Filter paper
4. Glass slide
5. Alcohol sponges.

Method

1. Clean the lobe of the ear or tip of a finger with alcohol and let dry.
2. For ear—glass slide is placed behind the ear lobe and held firmly in place. This provides a firm site for incision.
3. Pierce the lobe of the ear by a firm stroke against the glass slide (or pierce the fingertip). Discard the glass slide if ear lobe has been incised. Start the stop watch when the stab was made.
4. Bleeding of the wound should be allowed to proceed without pressure and the blood is allowed to drop on the filter paper. The paper should be moved so that each drop will fall on a fresh area. When bleeding slows, the wound is touched gently with a fresh area of the filter paper at 30 second intervals. When blood no longer stains the filter paper, the watch is stopped and the time recorded.

Normal Values

The normal range is up to 6 minutes. Between 6 and 10 minutes, the results are borderline. Over 10 minutes is definitely abnormal.

Precautions

1. In children heel should be used.
2. In suspected cases of a bleeding disorder, the bleeding may not be controlled easily from the ear lobe hence, fingertip puncture wounds are better.
3. The area to be punctured should not be congested.
4. The size and depth of the wound may vary if one does not have a standardized technique.
5. If bleeding persists for more than 15 minutes it should be stopped by placing a dry gauge sponges over the site and applying finger pressure (the filter paper used to collect the drops of blood can be dried and saved as a record of the procedure).

Ivys's Method

Preferred because of greater ease of standar-dization

Method

1. Cleanse the inner aspect of the forearm with spirit and let dry.
2. Place a blood pressure cuff on the upper arm, inflate at 40 mm Hg, and maintain the same throughout the test.
3. Select an area on the forearm—Volar aspect which is devoid of superficial veins. Stretch the skin laterally between the thumb and forefinger and hold in a taut position.
4. Take a cork, through which a no. 11 surgical blade has been inserted with the tip extending 3 mm beyond the cork surface (both cork and blade should have been sterilized before), the blade should be withdrawn from the cork and autoclaved before being used again.
5. Hold the cork with the thumb and forefinger of the free hand, and with the heel of the hand resting on the patient's arm, quickly make two skin punctures (actually they are small incisions) in the selected area. It is important that the surface of the cork meets the skin to ensure a 3 mm deep incision. Holding the skin taut prevents the test area from being depressed when the blade enters the skin.
6. Timing is begun as soon as the incisions are made and bleeding starts.
7. Using the edge of a piece of a filter paper to collect the blood, gently touch paper to the drop of blood which forms over the wound every 30 seconds. Do not rub or remove the clot. Do not touch the skin. Any disruption of formed fibrin or clot will prolong the bleeding time.
8. The bleeding time is reported when no blood stain is seen on the filter paper after a gentle touch. It is reported in intervals of 30 seconds. One can measure both wounds and average them, or take the reading of the last one to stop bleeding.

Normal Values

Normal values are 1–6 minutes. More than 6 minutes should be taken as abnormal.

Interpretation

1. Results of duplicate tests performed on the same individual should agree within 2–3 minutes at most.
2. Bleeding time is prolonged
   - When platelet count <100,000/mm^3
   - In patients on aspirin therapy
   - In acquired fibrinogen disorders

(If the platelets are young even in a thrombocytopenia patient, the bleeding time may not be raised as young platelets have enhanced hemostatic capabilities).

When platelet counts are low, one can calculate the expected bleeding time with the following formula:

$$\text{Bleeding time} = 30.5 \times \frac{\text{Platelet count/mm}^3}{3850}$$

A bleeding time longer than that calculated from platelet numbers alone, suggests defective platelet function in addition to reduced number. It is also possible to detect above-normal hemostatic capacity in cases in which active young platelets comprise the entire population of circulating platelets.

Clinical Implications

1. Bleeding time is prolonged when the level of platelets is decreased or when the platelets are qualitatively abnormal, as in
   a. Thrombocytopenia.
   b. Platelet dysfunction syndromes.
   c. Decrease or abnormality in plasma factors such as von Willebrand's factor and fibrinogen.
   d. Abnormalities in walls of the small blood vessels—vascular defects.
   f. Severe liver disease.
   g. Leukemia.
   h. Aplastic anemia.
   i. DIC disease.
2. Bleeding time can be either normal or prolonged in von Willebrand's disease. It will definitely be prolonged if aspirin is administered prior to testing.
3. A single prolonged bleeding time does not prove the existence of hemorrhagic disease because a larger vessel may have been punctured. The puncture should be done twice (on the contralateral side) and the average of the bleeding times can be taken.

Interfering Factors

1. The normal range may vary when the puncture is not of standard depth and width.
2. Touching the incision during the test will break off any fibrin particles and prolong the bleeding time.
3. Heavy alcohol consumption (as in alcoholics) may cause bleeding time to be increased.
4. Prolonged bleeding time will result from the ingestion of 10 g of aspirin up to 5 days before the test.
5. Other drugs that may cause the bleeding time to be increased include:
   - Dextran
   - Streptokinase—streptodornase
   - Mithramycin
   - Pantothenyl alcohol

Patient Preparation

1. Explain the purpose and procedure of the test to patient.
2. Warn patient not to consume aspirin for 5 days prior to test.
3. Advise patient not to consume alcohol in any form.

COAGULATION TIME

Capillary Tube Method of Wright

Blood is collected in about a dozen capillary tubes from a finger prick made after aseptic precautions. The tubes are sealed with plasticine and immersed in water bath at 37°C. After 4 minutes remove the first tube from the bath and expel the blood in it with one end immersed in a dish containing water. Repeat this every 30 seconds with the other tubes till the blood is expelled in a worm clot and note the time.

(An alternative way of determining the end point is to break the capillary tubes every 30 seconds until a clot is seen between the two broken ends). By these methods the normal clotting time is 5–10 minutes at 37°C and longer if performed at room temperature. This test should be avoided as tissue thromboplastin contaminates the oozing blood and hence, false reports may be obtained.

Lee and White's Method

Principle: Whole blood, when removed from the vascular system and exposed to a foreign surface, will form a solid clot. Within limits the time required for the formation of the solid clot is a measure of the coagulation system.

Requirements

1. Stop watch
2. Equipment for collection of blood
3. Clean, dry glass test tubes (10 × 75 mm)
4. Water bath (37°C)

Method

1. Make a clean venipuncture with as little trauma to (or time spent passing through) the connective tissue between skin and vein as possible. One may routinely or in selected cases use the two syringe technique, where—by one rinses the needle of all interstitial tissue fluid by drawing back 1 cc. of blood after entering the vein. Then remove the first syringe from the needle and quickly place on a second clean and dry syringe and drawback blood for the test.
2. Timing is begun when the blood first enters the syringe. The second syringe in the 'Two syringe' technique.
3. Draw 3–5 mL of blood and withdraw the syringe and needle. Disconnect the needle. Place approximately 1 mL of blood in each of three (10 × 75 mm) test tubes.
4. Place the tubes in a stand so that they remain upright and undisturbed, at room temperature for 10 minutes. If a 37°C water bath is available one may do the entire test at 37°C, and shorter clotting times will be found (if the test has been done at 37°C, do not wait for more than 5 minutes).
5. After 10 minutes (or 5 minutes) take the first of the tubes and gently tip it every 30 seconds to test for clotting. Do not tip it further than necessary to get the information.
6. When the first tube is clotted (can be inverted without blood running down the edge of the tube), record the time and start the tipping of the second tube every 30 seconds until it is also found to be clotted. Then do the same with the third tube (tipping is intended to allow one to ascertain when blood is clotted—not as a means of hastening clotting or of assuring mixing of the blood).

7. The time recorded for the clotting of the third tube is taken as the clotting time (the purpose of the first two tubes is to tell one when to start looking in the third tube, since the agitation of tipping does hasten the clotting). Some choose to tip the tubes in rotation (at 37°C) every 15 seconds, or tip all tubes at once, and average the results of the three tubes.

Normal Values

Normal times depend on method used. Normal range at 37°C is usually 5–10 minutes. Normal times at room temperature will vary with the degree of temperature present and the method used. If one uses the method which waits 10 minutes before starting to tip, then normal values may go as high as 22–25 minutes, especially in the cool season. Values shorter than 10 minutes should be suspected and the test repeated using the two-syringe technique to rule out contamination by tissue fluid (in the heat of April, May or June warm tropical climate blood will clot before 10 minutes without having been contaminated by tissue fluids). If one uses the method which waits 5 minutes before tipping begins, normal results are between 8–18 minutes. Longer than 20 minutes is abnormal. If clotting occurs in less than 7 minutes, the test should be repeated using two-syringe technique.

Precautions and Errors

1. The venipuncture must be without trauma to avoid contamination with tissue thromboplastin.
2. If all three tubes are clotted at 10 minutes (or 5 minutes) when one starts to tip the first tube, the test is unsatisfactory and should be repeated. If blood was drawn by single syringe technique, the most likely explanation is contamination, of the blood by tissue thromboplastin. If a two-syringe technique is used, it suggests that the patient's blood is hypercoagulable.
3. Vigorous agitation of the tubes will significantly shorten the coagulation time. So tipping should really be very gentle just to see if the blood has clotted.

Clinical Implications

1. Severe deficiencies of any of the coagulation factors must be present before the coagulation time will be prolonged. Fibrinogen for example, needs to be decreased to 50 mg/100 mL or less before the coagulation time is affected, the normal range of fibrinogen is 200–400 mg/100 mL.
2. When prothrombin is diminished to a level of 30% of normal, there will be a small change in coagulation time.
3. Prolonged coagulation time will be noted in afibrinogenemia and marked hyper-heparinemia.

Interfering Factors

1. *Quality of venipuncture:* The venipuncture must be carefully done because either tissue thromboplastin obtained as a contaminant when the venipuncture is done, or hemolyzed red blood cells suctioned when the blood is drawn, can cause a marked shortening of the coagulation time. The time required for a severe hemophiliac's blood to clot can be shortened from 1 hour to a normal value when a poor venipuncture is done.
2. *Type of test tube:* The coagulation time will be lengthened to 20–40 minutes if plastic or silicone coated test tubes are used.

3. *Drugs:* Increased coagulation time may be seen with
   - Mithramycin
   - Tetracyclines
   - Anticoagulants
   - Azathioprine
   - Carbenicillin

   Decreased coagulation time may be seen with
   - Corticosteroids
   - Epinephrine

CLOT RETRACTION

Principle

When whole blood is allowed to clot spontaneously, the initial coagulum is composed of all elements of the blood. With time the coagulum reduces in mass and fluid serum is expressed from the clot. This is due to an action of platelets on the fibrin network.

Requirements

1. Equipment for collecting blood
2. Clean, dry plain glass graduated centrifuge tube
3. Timer
4. Water bath 37°C.

Method

1. 5 mL blood is obtained with a standard twosyringe technique and transferred to the centrifuge tube.
2. Incubate it at 37°C in vertical position.
3. Record degree of retraction after 1, 2, and 4 hours. It may be necessary to loosen the clot gently from the wall of the test tube if contraction is not apparent at the end of 1 hour. The degree and rate of retraction should be noted. Note also any digestion of clot or discoloration of serum.

Clot retraction is directly related to platelet count; hence, it is impaired in thrombocytopenia, but is normal in hemophilia. In the method just described, one can remove the clot by using a hooked long needle and the volume of serum left behind can be measured. The percentage of clot can be calculated from the initial 5 mL of blood taken. In normal individuals, the clot percentage is about 50% at the end of one hour of the original blood volume taken.

Interpretation

- Patients with qualitative or quantitative platelet disorders have samples with scant serum and a soft, plump, poorly demarcated clot.
- The clot is small and serum voluminous if the patient has a low hematocrit.
- Patients with polycythemia have poor clot retraction because the large numbers of captured red cells separate fibrin stands and interfere with platelet contraction.
- If fibrinogen levels are low, the initial clot is so fragile that the delicate strands rupture and red cells spill out into the serum when retraction begins.
- Serum contamination by red cells is especially striking if fibrinolysis is abnormally brisk, as often happens with reduced fibrinogen levels. Sometimes in these cases, the incubated tube contains only cells and plasma with no fibrin clot at all.

Errors

1. When fibrinogen is reduced in amount, the clot may be very small and retraction may be interpreted as normal even though it is inadequate.
2. In the presence of active fibrinolytic activity, the clot may dissolve.
3. In normal blood the exuded serum will be clear and free of RBC's. The presence of significant number of RBCs in the serum suggests fibrinolytic activity.
4. With a low hematocrit value, the mass of the clot will be proportionately small and may give enormously high values.

SENSITIVE THROMBOPLASTIN REAGENT FOR PROTHROMBIN TIME (PT) DETERMINATION (ISI=1.0) UNIPLASTIN®

Courtesy: *Tulip Group of Companies*

Summary

The arrest of bleeding depends upon primary platelet plug formed along with the formation of a stable fibrin clot. Formation of this clot involves the sequential interaction of series of plasma proteins in a highly ordered and complex manner and also the interaction of these complexes with blood platelets and materials released from the tissues. Tissue thromboplastin, in the presence of calcium, is an activator, which initiates the extrinsic pathway of coagulation, which includes plasma coagulation factors VII, X, V, Prothrombin and Fibrinogen. During oral anticoagulant therapy most of the Vitamin K-dependent factors such as II, VII, IX, X, Protein C and Protein S are depressed, as also during the deficiencies of clotting factor activity which may be hereditary or acquired. Prothrombin Time determination is the preferred method for presurgical screening, as a liver function test, determination of congenital deficiency of factors II, V, VII and X and for monitoring of patients on oral anticoagulant therapy.

Reagent

Uniplastin is a novel, highly-sensitive, low opacity, ready to use liquid calcified Thromboplastin reagent is derived from rabbit brain. Each batch of reagent undergoes rigorous quality control at various stages of manufacture for its sensitivity and performance.

Reagent Storage and Stability

(a) Store the reagent at 2 to 8°C. *Do not freeze*, (b) The shelf life of the reagent is as per the expiry date mentioned on the reagent vial label. The uncontaminated reagent is stable for: 1 year at 2–8°C, 1 week at 18–25°C, 2 days at 37°C.

Principle

Tissue thromboplastin in the presence of calcium activates the extrinsic pathway of human blood coagulation mechanism. When *uniplastin* reagent is added to normal citrated plasma, the clotting mechanism is initiated, forming a solid gel clot within a specified period of time. The time required for clot formation would be prolonged if there is acquired or congenital deficiency of factors/factor activity in the extrinsic pathway of the coagulation mechanism or reduction in the activity of vitamin K- dependent clotting factors during oral anticoagulant therapy.

Note

1. *In vitro* diagnostic reagent for laboratory and professional use only. Not for medicinal use.

2. *Uniplastin* reagent is not from human source, hence contamination due to HBsAg and HIV is practically excluded.
3. Reagent contains 0.01% thimerosal as preservative.
4. It is very important that scrupulously clean and dry micropipette tips be used to aspirate/dispense the reagent.
5. Avoid exposure of the *uniplastin* reagent to elevated temperatures, contamination and undue stress due to high and low temperature exposure cycles. Immediately replace reagent cap after use and store at recommended temperatures only.
6. On prolonged storage at 2–8°C the thromboplastin suspension has a tendency to settle down. Homogenize the reagent by resuspending before use.

Additional Material Required

12 × 75 mm test tubes (plastic tubes are preferred), 0.1 mL and 0.2 mL precision pipettes, Stop watch, Water bath or heating block at 37°C, fresh normal plasmas for establishing MNPT.

Sample Collection and Preparation of PPP

Though no special preparation of the patient is required prior to sample collection by approved techniques, it is preferable that patients are not heavily exercised before blood collection. Fasting or only light nonfatty meals prior to blood collection provide samples with a desirable lower opacity. Withdraw blood without undue venous stasis or frothing into a plastic syringe fitted with a short needle of 19–20 SWG. The veinpuncture must be a 'clean' one and, if there is any difficulty, take a new syringe and needle and try another vein. Transfer the blood into anticoagulated tubes, after detaching the needle from the syringe. Do not delay mixing blood with anticoagulant. Avoid foam formation during mixing.

Mix exactly nine parts of freshly collected blood with one part of Trisodium citrate (0.11 mol/l, 3.2%). For occasional patients with hematocrit less than 20 % or greater than 55 %, this ratio must be readjusted to ensure valid results. Centrifuge immediately for 15 minutes at 1500–2000 rpm (approximately 1500 g) on a laboratory centrifuge and transfer the plasma into a clean test tube. It should be ensured that the plasma is free from platelets (PPP). Cap the test tubes to prevent deterioration of samples. Plasma must be tested preferably immediately. However, if the specimen are held at 22–24°C then they may be tested within 2 hours and if the specimen is held at 2–4°C, then they may be tested within 3 hours.

Test Procedure

Manual Method

1. Bring the reagent vial to room temperature (20–30°C). Mix the contents of the vial to homogenize the suspension completely.
2. Aspirate from the reagent vial enough reagent for immediate testing requirements in a thoroughly clean and dry test tube (Plastic test tubes are preferred).
3. Prewarm the reagent and bring to 37°C before use in test procedure (5–10 minutes may be required depending on the reagent volume to attain 37°C before testing).
4. Recap the reagent vial and replace immediately to 2–8°C.
5. To a 12 × 75 mm tube add 0.1 mL of plasma (PPP) and place the tube in a waterbath for 3–5 minutes at 37°C.

6. To the tube forcibly add 0.2 mL of *uniplastin* reagent (prewarmed at 37ºC for at least 3 minutes) and simultaneously start a stopwatch. Shake the tube gently to mix contents.
7. Gently tilt the tube back and forth and stop the stopwatch as soon as the first fibrin strand is visible and the gel/clot formation begins. Record the time in 'seconds'.
8. Repeat steps 4–6 for a duplicate test on the same sample.
9. Find the average of the duplicate test values. This is the prothrombin time (PT).

If a coagulation instrument is being used to perform the tests, the instrument manufacturers instructions must be strictly adhered to.

Calculation of Results

Manual Method

The results may be reported directly in terms of the mean of the double determination of PT of the test plasma in 'seconds'.

Or as a ratio 'R':

$$R = \frac{\text{Mean of the patient plasma PT in seconds}}{\text{MNPT for the reagent}}$$

Or as International Normalized Ratio (INR), INR = (R) ISI, where ISI = International Sensitivity Index of the reagent (Refer reagent vial label).

It is recommended by the WHO that MNPT should be established for each lot of PT reagents by each laboratory, since PT results are dependent on the combination of reagent lot, instrument and technique followed at each laboratory. Usually plasma from at least 20 normal healthy individuals should be used to establish the MNPT. The average of such PT results in seconds = MNPT.

Expected Values

Normal values using *uniplastin* are between 11 and 15 seconds. Between manual and Turbo densitometric instrument results a variation of 1–2 seconds may be expected. For photo optical instruments, it is recommended that each laboratory must establish its own normal range. It is mandatory that each laboratory must establish its own MNPT for each lot of *uniplastin*.

Oral anticoagulant therapeutic range: INR = 2.0–3.5

Remarks

1. It is recommended that controls with known factor activity should be run simultaneously with each test series to validate test run.
2. Incorrect mixture of blood and trisodium citrate, insufficient prewarming of plasma and reagent, contaminated reagents, glassware, etc. are potential source of errors.
3. Oxalated plasma may induce prolonged clotting times.
4. Since the PT test functions correctly only at 37 + 0.5°C, temperature of all equipment must be calibrated daily.
5. Clotting time of patients on anticoagulant therapy depends upon the type and dosage of anticoagulant and also the time lag between the specimen collected and the last dose.
6. Turbid, icteric, lipemic or grossly hemolyzed samples may generate erroneous PT results.

7. Glasswares and cuvettes used in the test must be scrupulously clean and free from even traces of acids/alkalies or detergents.
8. Plasma samples held at 4–8°C may undergo 'cold activation' leading to a marked shortening of the PT.
9. The PT may be shortened during acute inflammatory conditions which are accompanied by increase in fibrinogen levels and also by agents such as antihistamines, butabarbital, phenobarbital, caffeine, oral contraceptives and vitamin K. The PT may be prolonged by corticosteroids, EDTA, oral contraceptives, asparaginase, clofibrate, erythromycin, ethanol, tetracycline, aspirin and anticoagulants such as heparin and warfarin.
10. It is important that each laboratory expresses the results in terms of INR for patients on oral anticoagulant therapy for the clinician to adjust the dosage based on INR.
11. Since the test uses platelet poor plasma, each laboratory must calibrate the necessary force and time required during centrifugation to yield the PPP. Contamination of plasma with excess platelets could falsely elevate levels of some of the factors.
12. Homogenization of *uniplastin* reagent suspension before use is important to achieve accurate and consistent results.

APTT/PTTK CEPHALOPLASTIN REAGENT FOR PARTIAL THROMBOPLASTIN TIME (APTT) DETERMINATION USING ELLAGIC ACID AS ACTIVATOR LIQUICECIN-E®

Courtesy: *Tulip Group of Companies*

Summary

The arrest of bleeding depends upon primary platelet plug formed along with the formation of a stable fibrin clot. Formation of this clot involves the sequential interaction of a series of plasma proteins in a highly ordered and complex manner and also the interaction of these complexes with blood platelets and materials released from the tissues. Activated partial thromboplastin time (APTT) is prolonged by a deficiency of coagulation factors of the intrinsic pathway of the human coagulation mechanism such as factors XII, XI, IX, VIII, X, V, II and fibrinogen. Determination of APTT helps in estimating abnormality in most of the clotting factors of the intrinsic pathway including congenital deficiency of factor VIII, IX, XI and XII and is also a sensitive procedure for generating heparin response curves for monitoring heparin therapy.

Reagent

Liquicelin-E® is a liquid ready to use activated cephaloplastin reagent for the determination of APTT. It is a phospholipid preparation derived from rabbit brain with ellagic acid as an activator.

Each batch of reagent undergoes rigorous quality control at various stages of manufacture for its sensitivity and performance.

Reagent Storage and Stability

a. Store the reagent at 2–8°C. Do not freeze,
b. The shelf life of the reagent is as per the expiry date mentioned on the reagent vial label. The reagent is stable for: 1 year at 2–8°C, 1 week at 18–25°C, 2 days at 37°C.

Principle

Cephaloplastin activates the coagulation factors of the intrinsic pathway of the coagulation mechanism in the presence of calcium ions. APTT is prolonged by a deficiency of one or

more of these clotting factors of the intrinsic pathway and in the presence of coagulation; inhibitors like heparin.

Note

1. In vitro diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. Liquicelin-E®, reagent is not from human source hence, contamination due to HBsAg and HIV is practically excluded.
3. Reagent contains 0.01% thimerosal as preservative.
4. It is very important that clean and dry micropipette tips be used to dispense the reagent.
5. Avoid exposure of the reagent to elevated temperatures and contamination. Immediately replace cap after use and store at recommended temperatures only.

Sample Collection and Preparation

No special preparation of the patient is required prior to sample collection by approved techniques. Withdraw blood without undue venous stasis and without frothing into a plastic syringe fitted with a short needle of 19–20 SWG. The venipuncture must be a 'clean' one and, if there is any difficulty, take a new syringe and needle and try another vein. Transfer the blood into tubes, after detaching the needle from the syringe.

Mix exactly nine parts of freshly collected blood with one part of trisodium citrate (0.11 mol/L, 3.2%) or PROFACT available from TULIP; Centrifuge immediately for 15 minutes at 3000 rpm (approximately 2000 g) and transfer the plasma into a clean test tube. Plasma must be tested within three hours of blood collection. For heparin determination, platelet deficient plasma should be used, hence higher centrifugation time is required.

FNP Collection

Prepare a plasma pool (FNP) of freshly collected blood from at least five normal healthy donors and process as above. Plasma must be tested within three hours of blood collection.

Additional Material Required

(a) 12 x 75 mm test tubes (b) 0.1 mL, 0.2 mL and 2.0 mL precision pipettes (c) Stop watch (d) Water bath or heating block 37° C (e) Fresh normal pooled plasma (f) $CaCl_2$ (0.02 mol/l)

*Available From Tulip Diagnostics.

Test Procedure

Manual Method

1. Before use, the reagent should be mixed well by gentle swirling. Do not shake.
2. Aspirate from the reagent vial enough reagent for the immediate testing requirement in a thoroughly clean and dry test tube. Bring this reagent to room temperature before prewarming at 37°C for testing purposes.
3. Separate test tubes containing Liquicelin-E® and TULIP's calcium chloride solution should be brought to 37°C. (Depending on volume, approximately 5–0 minutes required). Do not incubate the test plasma.
4. To a 12 × 75 mm test tube, add 0.1 mL test plasma and 0.1 mL *Liquicelin*-E®. Shake tube briefly to mix the reagent and plasma, place tube at 37°C for 3–5 minutes.
5. Following incubation period, add forcibly 0.1 mL prewarmed calcium chloride into the plasma and Liquicelin-E® mixture, simultaneously start a stop watch. Shake tube briefly to mix contents, keep at 37°C for 20 seconds.

6. Following 20 seconds incubation, remove the tube, gently tilt back and forth until a gel clot forms, stop the watch, record time.
7. Repeat steps 2–4 for a duplicate test using the same test plasma.
8. Find the average from the duplicate test values. This is the activated partial thromboplastin time (APTT of patient plasma).
9. Similarly repeat steps 2–4 twice, and record duplicate values using FNP in place of test plasma (APTT of FNP).

If a coagulation instrument is being used to perform the tests, the instrument manufacturers instructions must be strictly adhered to.

Calibration curve method (For determination of heparin concentration):

1. Dilute heparin (as used for treatment) with physiological saline to a concentration of 10 U/mL.
2. Mix 0.2 mL of 10 U/mL diluted heparin with 1.8 mL of FNP to give a heparin standard of 1 U/mL concentration.
3. Dilute the heparin standard as prepared above (1 U/mL) with FNP as follows:

Test tube No.	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Heparin standard (1 U/mL)	0.5	0.4	0.3	0.2	0.1	0.1	–
FNP in mL	–	0.1	0.2	0.3	0.4	0.9	0.5
Heparin concentration (U/mL)	1	0.8	0.6	0.4	0.2	0.1	0.0

4. Pipette 0.1 mL each of the seven heparin dilutions into clean test tubes.
5. Add 0.1 mL Liquicelin-E® reagent to each test tube.
6. Mix well and incubate each test tube at 37°C for exactly 3 minutes before testing.
7. Forcibly add 0.1 mL calcium chloride (prewarmed at 37°C) to each test tube, one by one and simultaneously start the stopwatch.
8. Gently tilt the tube back and forth and stop the stopwatch as the first fibrin strand is visible and the gel/clot formation begins. Record the time in seconds.
9. Repeat steps 4–8 for each dilution for duplicate test, and find the average of the duplicate test values.
10. Plot the mean of the double determination in 'seconds', against each heparin concentration using Liquicelin-E® graph paper.
11. Clotting times (APTT) of test specimens can be interpolated against the heparin concentration to determine the heparin concentration of the sample in U/mL.

Calculation and Reporting of Results

Manual Method

a. The result may be reported directly in terms of the mean of the double determination of the APTT of the test plasma or
b. As a ratio R as follows:

$$R = \frac{\text{APTT of patient plasma (in seconds)}}{\text{APTT of FNP (in seconds)}}$$

Calibration Curve Method

Heparin concentration in the test sample can be directly obtained from the Liquicelin-E® calibration curve by interpolating the test plasma clotting time against the heparin concentration in U/mL.

Expected Values

Normal values using Liquicelin-E® reagent are between 21 and 29 seconds at 3 minutes activation time. Between manual and turbo-densitometric instrument results a variation of 1–2 seconds may be expected. For photo optical instruments, it is recommended that each laboratory must establish their own normal range.

Remarks

1. Due to inter and intralaboratory variations users must establish their own normal population range as well as normal and abnormal range.
2. It is recommended that controls with known factor activity should be run simultaneously with each test series routinely.
3. Incorrect mixture of blood and trisodium citrate, insufficient prewarming of plasma and reagent, contaminated reagents, glassware, etc. are potential source of errors.
4. Incorrect dilutions of heparin is also a potential source of error.
5. Oxalated plasma may induce prolonged clotting times.
6. Clotting time of patients on anticoagulant therapy depends upon the type and dosage of anticoagulant and also the time lag between the specimen collected and the last dose.
7. Abnormalities of coagulation factor VII, factor XIII and platelets are not detected by this test procedure.
8. For automated equipment it is strongly recommended that the equipments manufacturers methodology is strictly adhered to.
9. In heparin monitoring time of collection of blood sample is important since the *in vivo* half-life of heparin is approximately 1.5 hours. When it is administered intravenously it has an immediate anti-coagulant effect but its efficacy decreases rapidly with time.
10. Platelet factor IV, a heparin-neutralizing factor can be released due to platelet aggregation or damage. In order to prevent this phenomenon in vitro the specimen should be collected with a minimum of trauma.
11. Decrease in APTT time is observed in males under estrogen therapy and oral contraceptive administration in females.

Fibrinolytic Activity

The three methods presented below are measures of fibrinolytic activity in general and are influenced by many factors. These serve as screening procedures, but the specific contribution of the various factors must be determined by other means. The lack of suitable standards makes quantitative measurements unavailable for most diagnostic laboratories.

Euglobulin Lysis Time

Principle

Euglobulin fraction of plasma contains fibrinogen and all the plasminogen activator and plasminogen of plasma but only traces of the antiplasmins. The lysis of a fibrin clot formed by the addition of thrombin is a measure of the fibrinolytic activity.

Requirements

1. Equipment for collection of blood
2. Centrifuge
3. Topical thrombin
4. Serological pipettes
5. Carbon dioxide. A tank of CO_2 fitted with a valve to allow control of the rate of flow.

Method

1. Blood is collected in the usual manner and mixed immediately with 0.11 M sodium citrate in a ratio of 1 part citrate solution to 9 parts blood.
2. Plasma is obtained by centrifugation.
3. 0.4 mL plasma is placed in a test tube and 7.6 mL. distilled water is added.
4. CO_2 is bubbled into the solution through a capillary tube for 30 seconds.
5. The precipitate which forms is collected by centrifugation at about 3,000 rpm for 15 minutes.
6. The precipitate is dissolved in 1 mL M/15 phosphate buffer, pH 7.2.
7. To the euglobin in phosphate buffer, 0.1 mL thrombin (topical thrombin diluted to 100 units per mL with saline) is added. The solution is mixed.
8. Clotting should be rapid. After clotting has occurred, the tube is placed in water bath (37°C) and observed for lysis of clot, which is the end-point.

Result

In normal plasma a period of 2–4 hours is required for euglobin clot lysis to occur. (The technique should be standardized in each laboratory).

DILUTE BLOOD CLOT LYSIS TIME

PRINCIPLE

Plasmin inhibitors lose activity on dilution to a greater extent than fibrinolytic activity. Whole blood is diluted with a buffer solution and clotted by the addition of thrombin. The clot is observed for lysis of the clot.

Requirements

1. Equipment for collection of blood sample
2. Test tube
3. Timer
4. Phosphate buffer, pH-7.4. To 1000 mL distilled water, 9.47 g Na_2HPO_4 is added and dissolved. This is mixed with 250 mL distilled water containing 3.02 g KH_2PO_4
5. Topical thrombin (or any other make) diluted to 100 units per mL with normal saline.

Method

1. Tubes containing 1.70 mL buffer and 0.1 mL. thrombin solution are placed in an ice bath.
2. Collect blood sample in standard manner using a syringe that can deliver accurately 0.2 mL aliquots of blood.
3. Add 0.2 mL blood to each of two tubes containing buffer and thrombin and mix.
4. Clotting should occur promptly.
5. Tubes are placed in refrigerator (4°C) for 30 minutes and then transferred to a water bath at 37°C.
6. The clots are observed for lysis. The endpoint is fragmentation rather than complete dissolution of the clot.

Result

Blood from a normal subject should not lyse in less than 6–10 hours. The test is qualitative. If results indicate rapid lysis of the clot, more quantitative methods are necessary.

FDPS: A QUALITATIVE AND SEMIQUANTITATIVE LATEX SLIDE TEST FOR DETECTING CROSS-LINKED FIBRIN DEGRADATION PRODUCTS IN HUMAN PLASMA X-L FDP

Courtesy: *Tulip Group of Companies*

Summary

During coagulation sequence of reactions occur in the body in response to variety of external and or internal stimuli. The enzymatic cascade reaction terminates in the conversion of *firbrinogen* to *fibrin*, by the enzyme *thrombin*.The fibrin gel is then converted to a stable fibrin clot by thrombin activated factor XIII.

Finally, the fibrin network is dissolved by the enzyme *plasmin* to generate cross-linked fibrin degradation products (XL FDP). D dimer comprising of two D fragments cross-linked together, is the smallest plasmin resistant molecular unit present within XL FDP. Detection of D dimer is invaluable as a diagnostic marker for thrombotic conditions such as disseminated intravascular coagulation (DIC), deep vein thrombosis (DVT) and pulmonary embolism (PE). D dimer levels can also be used to monitor thrombolytic therapy with tPA and with streptokinase, thrombotic complications in pregnancy, acute myocardial infarction, sickle cell crisis, severe septic infections, liver disease, DIC accompanying snake bite and prognosis and response to therapy in cancer.

Reagent

1. XL FDP reagent: A uniform suspension of polystyrene latex particles coated with mouse monoclonal anti D-dimer Antibody (DD-3B6/22). The reagent is standardized to detect XL FDP >200 ng/mL.
2. Positive control, reactive with XL FDP latex reagent.
3. Negative control, nonreactive with XL FDP latex reagent.
4. Phosphate buffer, for performing semiquantitative test.

   All the reagents contain 0.1% sodium azide as preservative.

Each batch of reagents undergoes rigorous quality control at various stages of manufacture for its specificity, sensitivity and performance.

Reagent Storage and Stability

1. Store the reagent at 2–8°C. Do not freeze.
2. The shelf life of the reagent is as per the expiry date mentioned on the reagent vial labels.

Principle

XL FDP slide test for detection of cross-linked fibrin degradation products is based on the principle of agglutination. The test specimen (plasma) is mixed with XL FDP latex reagent. The sensitivity of the reagent is ~200 ng/mL, below which samples are negative and above which samples give a positive agglutination reaction.

The cross-linked fibrin degradation products, D dimer, D dimer E, and high molecular weight derivatives are all recognized by TULIP XL FDP reagent incorporating the monoclonal antibodies. No binding was found to the fibrinogen degradation products XYD and E to 20 mg/L or to fibrinogen up to 1000 mg/L.

Note

1. *In vitro* diagnostic reagent for laboratory and professional use only. Not for medicinal use.
2. The reagents contain 0.1% sodium azide as preservative. Avoid contact with skin and mucosa. On disposal flush with large quantities of water.

3. All the reagents derived from human source have been tested for HBsAg and Anti-HIV antibodies and are found to be non-reactive. However, handle the material as if infectious.
4. The reagent can be damaged due to microbial contamination or on exposure to extreme temperature conditions. It is recommended that the performance of reagent be verified with positive and negative controls supplied with the kit.
5. Shake the XL FDP latex reagent vial before use to disperse the latex particles uniformly and improve test readability.
6. Only a clean and dry glass slide must be used. Clean the slide with distilled water and wipe dry.

Sample Collection and Preparation

No special preparation of the patient is required prior to sample collection. Plasma samples are recommended for use with XL FDP test. Fresh EDTA, citrate or heparinized anticoagulated plasma specimens are suitable for performing the test.

Sample storage:	20–25°C	—	8 hours
	2–8°C	—	4 days
Frozen	(–20°C)	—	2 months

Thaw frozen specimens at 37°C and centrifuge plasma before testing.

KIT Composition

1. XL FDP latex reagent, positive control, negative control, PBS buffer.
2. Glass slide with six reaction circles, disposable sample dispensing dropper, mixing sticks, rubber teat, package insert.

Additional Material Required

Stop watch, test tubes high intensity direct light source.

Test Procedure

Bring all the reagents and sample to room temperature before performing the test.

Qualitative Method

1. Pipette one drop of plasma specimen onto the glass slide using the disposable dropper provided with the kit. Hold the dropper exactly in vertical position to dispense the drop accurately.
2. Add one drop of XL FDP latex reagent adjacent to the drop of plasma specimen, taking care to hold the dropper in a vertical position while dispensing the drop. Do not let the dropper tip touch the plasma specimen on the slide.
3. Using a mixing stick, mix the plasma and latex reagent uniformly over the entire circle.
4. Immediately start a stop watch, rock the slide gently, back and forth, and observing for agglutination macroscopically at three minutes.
5. Do not read the test result beyond three minutes.

Semiquantitative Method

1. Using PBS buffer solution prepare serial dilutions of the plasma sample 1:2, 1:4, 1:8, 1:16, 1:32 and so on.
2. Pipette each dilutions of plasma specimen on to the separate reaction circles.

3. Add one drop of XL FDP latex reagent to each drop of diluted plasma specimen on to the slide. Do not let the dropper tip touch the diluted plasma specimen on the slide.
4. Immediately start the stop watch. Rock the slide gently, back and forth, observing for agglutination macroscopically at three minutes.

Interpretation of Results

Qualitative Method

- Agglutination is a positive result indicating D dimer level above 200 ng/mL.
- No agglutination is a negative result indicating absence of clinically significant D dimer levels in the plasma specimen.

Semiquantitative Method

Agglutination in the highest plasma dilution corresponds to the approximate amount of D dimer level in ng/mL.

To calculate D dimer level in ng/mL in the sample, use the following formula:

- D dimer level (ng/mL) = 200 × d
- d = highest dilution of plasma showing agglutination during the semi-quantitative test of the sample.

NB: Activation of the coagulation system with subsequent microvascular fibrin deposition and lysis has been reported in diverse clinical conditions such as trauma, surgery, inflammation and malignancy. Elevated levels of plasma XL FDP may be expected to occur in such conditions.

Remarks

1. D dimer half-life is approximately 6 hours in circulation of individuals with normal renal function. Patients with stabilized clots and not undergoing active fibrin deposition and plasmin activation may not give detectable D dimer elevations.
2. In PE, the larger the clot size, higher the expected level of circulating D dimer. Conversely, the amount of D dimer released from very small clots may be diluted by the circulation and may not give a detectable increase.
3. Fibrinolysis is a highly regulated process and in delicate dynamic balance. In case of hereditary, acquired deficiency and dysfunction of fibrinogen, the rate of fibrinolysis will be altered thereby not giving a detectable D dimer level.
4. As with any laboratory test, detection of elevated levels of XL FDP in a specimen should be correlated with clinical findings.

AUTOMATION IN COAGULATION ANALYSIS

HEMOSTAR

Sleek, user-friendly coagulation analyser based on turbo-phototometric detection of clotbased assays. Hemostar has 10 in-built incubation positions for Sample and 1 location for reagents (Fig. 24.14).

- It allows the user to make all baisc tests for the analysis of plasmatic hemostatic phase, e.g. Quick, test (PT), aPTT, TT, fibrinogen, defective factors, etc.
- It is a totally open instrument to be used with all kinds of quality reagents, simply by following the producers instructions.
- It includes a magnetic stirrer system in order to mix well the reagents and plasma.

- *Optical group:* Made up of a Wolfram lamp at low voltage and long life, an optical light pass, the reaction cell holder and a solid state photocell which registers all absortion changes produced.
- *Thermostatic block:* Thermostated device at 37°C + 0.1°C with magnetic stirrer motor built-in
- *Screen:* Four digit indicators with tenth of second precision, thermostatic block control temperature LED (37°C) and two operation keys, the chronometer can be used seperately to control incubation time.
- *Tests:* Quick Test (PT), aPTT, TT, Fibrinogen, deficiency factors, etc.
- Power requirements: AC 220 V. 50 Hz 120 VA
- Dimensions: 180 × 300 × 140 nm
- Weight: 2, 3 kg

Fig. 24.14: Hemostar
(*Courtesy: Tulip Group of Companies*)

Technical Features

System	• Automatic single channel coagulometer based on turbophotometric clot detection system
Measurement	• Optomechanical measuring system • Automatic start of measurement on addition of reagent/sample for precise result • On screen digital chronometer to measure tenth of a second accurately
Sensitivity	• Highly-sensitive in all measuring range because of optomechanical measuring system (combination of optical controls with mechanical movement of magnetic stirrers)
Incubator	• Inbuilt incubator (solid state) fixed at 37°C • Incubator lodges 10 reaction cuvettes and one reaction vial
Testing mode	• PT, APTT, TT, fibrinogen and factor assays.
General	
Lamp source	Low voltage tungsten lamp
Detector	IR detector
Motor	For stirring action of magnetic stirrer
Display	Back illuminated LCD with single row and 14 characters
Power supply	220V, 50 Hz/60 Hz
Power consumption	50VA
Serial output	RS 232 standard
Working temperature	15°–35°C
Dimensions	18 × 30 × 40 cm
Weight	2.2 kg

HEMOSTAR XF

Single channel coagulometer with turbo-photometric clot detection principle with an optomechanical measuring system which improves sensitivity of detecting weak fibrin polymers. Hemostar XF has 10 open locations which enables to perform PT, APTT, TT, fibrinogen assays and factors assays. This walk away plug and play system has been designed to make complicated clotting analysis of hemostasis system into a simple error free task (Fig. 24.15).

- Single-channel coagulometer, fully-controlled by built-in microprocessor.
- Turbophotometric detection of fibrin polymer formation (clots)
- Solid state thermostat set at 37°C
- Keyboard for programming and data entry.
- Digital control and results display.
- Integral printer for easier transcription of results
- Enables performance of all types of hemostasis plasma phase studies and particularly: Quick, a PPT, TT, firbrinogen, factor studies.
- Optics: Low voltage photometry lamp
- Truncated cone reading cell
- Photofeed detection
- Magnetic stirrer motor
- Thermostat: Metal block thermostatted to 37°C ± 0.1°C by solid state elements
- Capacity for 10 cells and a reagent bottle

Fig. 24.15: Hemostar XF
(*Courtesy: Tulip Group of Companies*)

- Programming: The *clot* 1A has a permanent memory for programming the parameters for each coagulation technique. These parameters remain stored in the memory until they are off reprogrammed, even when the unit is switched.
- The keypad enables the user to sequentially access the programming and speeds up date entry during routine use of the instrument.
- The digital display functions as a programming and results monitor.
- Results: Displayed on the screen and in printout form, containing all parameters and the relevant identification number.
- Size: 30 × 35 × cm
- Weight: 3,700 kg
- Power supply: 220 V 50 Hz 100 VA

Technical Features

System	• Microprocessor controlled automatic single channel coagulometer based on turbo-photometric clot detection system
Measurement	• Optomechanical measuring system • Automatic start of measurement on addition of reagent/sample for precise result • On screen digital chronometer to measure tenth of a second accurately
Sensitivity	• Highly-sensitive in all measuring range because of optomechanical measuring system (combination of optical controls with mechanical movement of magnetic stirrers)

Contd.

Incubator	• Inbuilt incubator (solid state) fixed at 37°C • Having 19 incubation positions
Testing mode	• PT, APTT, TT, fibrinogen and factor Assays
General	
Programming	10 locations for programming parameters
Keyboard	Keyboard for programming and data entry
Printer	Built in quiet thermal printer for direct reporting of result in INR, percent Activity, PTR and APTT ratio
Serial output	RS 232 standard
Lamp Source	Low voltage tungsten lamp
Detector	IR detector
Motor	For stirring action of magnetic stirrer
Display	Back illuminated LCD with 4 rows of 20 characters
Working temperature	15–35°C
Power supply	220V, 50 Hz, 60 Hz
Power consumption	100 VA
Dimensions	31 × 15 × 35 cm
Weight	3.7 kg

COALAB 6000

Fully-automated compact and versatile bench top coagulation analyzer for clotting assays based on the patented turbodensitometric principle of clot detection. CoaLAB 6000 is engineered to operate in random access mode for PT, APTT and fibrinogen with an option for batch and stat mode. CoaLAB 6000 has the agility for throughput of 130 PT tests per hour. CoaLAB is an ideal coagulation analyzer designed to simplify complicated coagulation analysis into a walkway task for laboratories with high and medium throughput (Fig. 24.16).

Compact

- Bench top coagulation analyzer
- Built-in computer with display and keypad
- Internal Thermal Printer
- On board waste box for used cartridges

Versatile

- Single and double determination adjustable
- Primary and secondary tubes can be used
- All reagent positions can be stirred
- On or off switch option allowing real time or patient oriented printing of results
- Stat-Program
- Automatic reagent level control
- All test parameters can be reprogrammed and/or changed by the user

Fig. 24.16: CoaLAB 6000
(*Courtesy: Tulip Group of Companies*)

User Friendly

- Easy installation of the analyser
- Optimal user guidance through routine software
- Easy method change via chip cards
- Optimized cuvette cartridge consumption
- Preprogrammed method settings and evaluation procedures for: PTT, aPTT and fibrinogen assays
- Updating and/or upgrading via chipcards

Technical Specifications

Parameters	*Specifications*
CoaLAB 6000	Fully Automated coagulation analyzer for clotting assays
Measuring principle	Turbodensitometric. This optomechanical principle is able to measure lipemic and turbid plasma samples and reagents containing kaolin.
Measuring station	6 measuring channels (parallel working mode)
Loading station	18 sample positions (for primary and special cups) 6 reagent positions (for 4 mL/10 mL vials) at room temperature. All reagent positions can be stirred. 6 positions for cuvette cartridges, each cartridge includes 6 cuvettes.
Cuvette	Cuvette cartridges with 6 cuvettes including mixers, reagent vials, sample-cups. Test volume: max, 250 µL
Traverse	Movement into 2 directions X-Z, for pipetting and transport of plasma and reagent Pipettor heated to 37°C. A level sensor detects the presence of liquid.
Dilutor	250 µL syringe, 1 pipetting tube
System liquid	Distilled water for washing and cleaning of pipettor
Washing solution	Special washer to avoid carry-over of thrombin.
Display	Alphanumeric LCD-Display, 2 lines, 20 characters each.
Operating mode	Random access mode for PT/aPTT and fibrinogen, batch mode selectable
Throughput	>120 PT tests/hour
Printer	Built-in thermal printer
Printout	Patient and result oriented (selectable)
Barcode reader	Optional, for fast introduction of patient/sample numbers.
Interfaces	1 × RS232 C Host/Service, 1 × Barcode Scanner
Power supply	100–240 V, 57–63 Hz, 150 VA
Dimensions	72 × 45 × 55 cm (~28.3 × 17.7 × 21.7 in)
Weight	17 kg (~ 35 pounds)
Software features	Optimal user guidance through routine software. Method menu for PT, aPTT and fibrinogen. Additional assays evaluated on the analyzer: Factors II, V, VII, VIII, IX, X, XII and Thrombin Time. Documentation of all test parameters, e.g. calibration curves, date, time, conversions of percent, g/L, mg/dL, Ratio, INR and incubation time can automatically be loaded via ChipCARD. Easy method change via ChipCARD Two software languages (German/English) on board Consecutive award of sample/patient ID's or by optional barcode scanner or manually by user External service and research program.

Contd.

Parameters	*Specifications*
Special features	Single and double determination adjustable On board waste box for used cartridges, for hygienic disposal of used cuvette cartridges. Plasma or reagent start (can be configured) Optimized cuvette cartridge consumption to avoid waste of unused cuvettes. On or off switch option allowing "real time" or patient oriented printing. User oriented result management (print-out, LCD and Host). Advanced paper feed button. Wide range power supply 100–240 V, 47–63 Hz
Safety features	Easy installation of analyzer ("plug and play") Highlighted Status/Error Messages in printout. Special check of liquid level in washing station. Optimized liquid check at pipettor. Optimized cuvette cartridge guidance. Software controlled cuvette cartridge detection. Acoustic status signals of analyser for convenient user information. Easy change of analyzer tubings.

TROUBLESHOOTING

GENERAL INSTRUCTIONS FOR COAGULATION TEST

Possible causes	*Solutions*
Preparation of patients	
1. Patients heavily exercised before blood colletction.	Although no special preparation of patients is required prior blood collection, fasting patients or patients on a light non-fatty meal are preferable. Fasting samples collected provide desirable lower opacity, which improves the sensitivity of clot detection especially when photo optical instruments are being used
2. Erroneous results are obtained due to varying opacity	Turbid, lipemic or grossly hemolyzed samples vary in opacity and therefore generate erroneous results

Sample Collection Techniques

Possible causes	*Solutions*
1. Frothing of blood	Blood should be withdrawn without undue venous stasis and without frothing into a plastic syringe with a short needle of 19–20 SWG. The venepuncture must be a 'clean' one besides the tourniquet should not be placed too tightly or for extended lengths of time. Patting the venepuncture site should also be avoided.
2. Delay in mixing blood with anticoagulant	Distribute blood into test tubes after detaching the needle from the syringe. Mix blood with the anticoagulant immediately by gentle inversion of the tube
3. Formation of microclots leading to artificially prolonged results	Clean venepuncture is essential to avoid formation of microclots at the site of venepuncture and consumption of factors, which will lead to artificially prolonged results
4. Use of improper needle for withdrawal of blood	With smaller bore longer needles blood will remain in contact with metal surface for a longer time leading to initiation of clotting or partial consumption of factors being assayed causing error in results. Therefore short bigger bore needles are recommended since it allows free flow of blood within the syringe and reduces blood contact with metal surface

Sample Preparation

1. Choice of anticoagulant	The anticoagulant used for most coagulation procedures is sodium citrate or preferably buffered sodium citrate
2. Shift in the ratio of citrate to blood	The optimum ratio of citrate to blood is 1 part of anticoagulant to 9 parts of blood • *More blood, less citrate:* Leads to formation of clots, consumption of factors and subsequent prolongation of results during test • *More citrate, less blood:* Consumption of calcium from the reagents giving prolonged test results

Sample Processing and Storage

1. Improper containers used for blood collection and processing	Containers for blood collection and processing should be clean an dry, free of detergents; acids and alkalies ideally made of plastic or siliconized glass tubes
2. Samples used for a long time are used for testing	Fresh samples should be used for testing. If specimen are kept at 22–24°C, then they must be tested within 2 hours and kept at 2–4°C, then within 3 hours
3. Sample not stored properly	Samples stored for future testing should be capped tightly to ensure accuracy of results
4. Centrifugation time and speed not maintained	Excessive centrifugation may destroy clotting factors due to heat generation. Maintain proper centrifugation speed and time to avoid erroneous results

Calibration of Instruments/Equipments

Possible causes	*Solutions*
1. Water baths not preset properly	To achieve accuracy and reproducibility, preset waterbath at 37 ± 0.5°C
2. Improperly calibrated pipettes	Prior to testing, check whether the pipettes so used are being regularly calibrated or not

Storage and Procedure

1. Deterioration of reagents due to thermal stress and high ambient temperatures	Most coagulation reagents are extremely delicate. For them to maintain their sensitivity and performance over the usage period recommended storage temperatures must strictly adhered to
2. Error in performing the test procedure	Ensure that the procedure and addition sequence is followed as indicated in the package insert

Critical Requirement of MNPT in the Derivation of INR

MNPT should ideally be derived by each laboratory from 20 or more normal patients for a given PT reagent and lot under use. If "normal control plasmas" are used in place of patient plasma for arriving at MNPT, it can effect the evaluation of the patients' level of anticoagulation.

ISI Value of PT Used and Method of Clot Detection

INR loses some precision when comparisons are made with thromboplastins with different ISI values. ISI values should be adapted to the methods used for clot detection ideally close to 1.0 as possible.

INR SYSTEM

Accuracy of the INR system depends on:
- The INR system effectiveness would still depend on the calibration of the coagulation instruments as well
- as thromboplastin reagents used.
- Derivation of the correct MNPT and use of mean normal range in each laboratory.
- Usage of thromboplastin reagents with ISI of preferably 1.0 or as close to 1.0 as possible.
- The correct use of formula to compute the INR.
- Uniform understanding of the INR system by clinicians as well as laboratarians.

PROTHROMBIN TIME

Uniplastin®/Liquiplastin®/Lyoplastin®

Problem: Prolonged Clotting Time

Possible causes	*Solutions*
1. Oxalated plasma may be used	Factor V and Factor VII are more labile in sodium oxalate. Buffered 3.2% sodium citrate is an ideal anticoagulant since Factor V and Factor VII are more stable in citrate. Therefore, citrate plasma should be used.
2. Plasma not tested immediately after blood collection	Plasma must be tested within 3 hours of blood collection
3. Insufficient prewarming of the plasma and reagents	Plasma should be incubated for 3–5 minutes at 37°C and the reagents should be incubated for at least 10 minutes at 37°C before commencing the testing procedure. Do not incubate the entire vial. Incubate only the requisite quantity
4. Incorrect mixture of blood and sodium citrate. The concentration of sodium citrate, which is used for the test, may be incorrect	Ensure that nine parts of freshly collected blood are mixed with one part of sodium citrate for normal hematocrit or PCV. For occasional patients with PCV less than 20% (e.g., Microcytic hypochromic anemia) and greater than 55% (e.g., polycythemia vera), the anticoagulant to blood ratio must be readjusted using the formula, $C = 1.85 \times 10^{-3} (100\text{-}H)V$ where, C = volume of sodium citrate in mL V = volume of whole blood + sodium citrate in mL H = Hemocrit in percentage
5. The manufacturer's test protocol not followed in case of automated instruments	Adhere to the instrument manufacturer's instructions and test protocol
6. Temperature of water bath not set correctly	The temperature of the water bath should be preset at 37°C ± 1°C
7. Expired reagents are used for testing	Check the expiry date of the reagents before use
8. Incorrect addition of reagent reagent	The test requires extreme precision, besides the addition of the should be followed as per the instructions given in the package insert. Exactly 0.1 mL of plasma and 0.2 mL of liquiplastin/uniplastin reagent prewarmed at 37°C should be used. Well-calibrated micropipettes should be used for dispensing
9. Contaminated reagents and glassware used for testing	Check the working of the reagents with normal control plasmas. Check the reagents for turbidity. Ensure that clean and dry glassware and micropipettes are used

Contd.

Possible causes	*Solutions*
10. Contaminated/wet micropipette tips used for retrieving the reagent	Ensure that clean and dry tips are used for retrieving the reagent
11. Clotting times of patients on anticoagulant therapy depends on the type and dosage of anticoagulants and also on the time lag between specimen collection and last dose of anticoagulant	The history of the patient must be taken into consideration to determine the anticoagulant used as well as the time lag between specimen collection and last dose of anticoagulant
12. Error in reading and interpretation of test results: a. The stopwatch is not stopped as soon as the gel clot is seen b. Clot formation is observed in inadequate light c. Water droplets are present at the base of the tube	The test protocol should be adhered to. The results should be read and interpreted as per the test protocol. The stopwatch should be stopped as soon as the gel clot is seen since there is a difference of 2–3 seconds between the beginning and end of clot formation. There should be adequate light while observing for clot formation. Wipe the base of the tube to remove additional water as it hampers reading of the test results
13. Improper storage condition leading to reagent precipitation	To maintain the sensitivity and performance of the reagent, avoid thermal stress due to exposure to high ambient temperature thereby causing precipitation, ensure that the reagent is stored at the recommended temperature
14. Improper mixing of the reagent after prolonged storage at 2–8°C	On prolonged storage at 2–8°C, thromboplastin suspension tends to settle down. Homogenize the reagent by resuspending before use
15. In case of automated instruments, the entire reagent vial is incubated thereby leading to deterioration of the reagent on repeated incubation	Only the requisite quantity of the reagent for performing the test should be incubated

Problem: Shortened Clotting Time

Possible causes	*Solutions*
1. Broken glassware used allowing silica to trigger the clotting reaction	Ensure that broken glassware is not used for testing purposes
2. Administration of drugs/other clinical conditions	PT tests are shortened because of the administration of drugs such as antihistamines, butabarbital, phenobarbital, caffeine, oral contraceptives and vitamin K. It is therefore essential to know the patient history to determine the type of anticoagulant used and the time lag between the last dose of the anticoagulant and specimen collection

Problem: Prolonged Clotting Time

Possible causes	*Solutions*
1. Oxalated plasma may be used	Factor V and factor VII are more labile in sodium oxalate. Buffered 3.2% sodium citrate is an ideal anticoagulant since factor V and factor VII are more stable in citrate. Therefore citrate plasma should be used
2. Plasma not tested immediately	Plasma must be tested within 3 hours of blood collection after blood collection
3. Insufficient prewarming of the plasma and reagents	Plasma should be incubated for 3–5 minutes at 37°C and the reagents should be incubated for at least 10 minutes at 37°C. Do not incubate the entire vial. Incubate only the requisite quantity

Contd.

Possible causes	*Solutions*
4. Incorrect mixture of blood and sodium citrate. The concentration of sodium citrate, which is used for the test, may be incorrect	Ensure that nine parts of freshly collected blood are mixed with one part of sodium citrate for normal hematocrit or PCV. For occasional patients with PCV less than 20% (e.g., microcytic hypochromic anemia) and greater than 55% (e.g., polycythemia vera), the anticoagulant to blood ratio must be readjusted using the formula, $C = 1.85 \times 10^{-3} (100\text{-}H)V$ where, C = volume of sodium citrate in mL V = volume of whole blood + sodium citrate in mL H = Hemocrit in percentage
5. Molarity of calcium chloride	Check the molarity of the calcium chloride being used. It should be 0.025 M
6. Contaminated/wet micropipette tips used for retrieving the reagent	Ensure that clean and dry tips are used for retrieving the reagent
7. The mixture of plasma and reagent is not incubated for minimum time period of 3 minutes	0.1 mL of plasma and 0.1 mL of Liquicelin-E reagent should be mixed well and incubated for exactly three minutes at 37°C for effective contact activation and to obtain accurate results
8. Error in reading and interpretation of test results. a. The stopwatch is not stopped as soon as the gel clot is seen b. Clot formation is observed in inadequate light c. Water droplets are present at the base of the tube	The test protocol should be adhered to. The results should be read and interpreted as per the test protocol. The stopwatch should be stopped as soon as the gel clot is seen since there is a difference of 2–3 seconds between the beginning and end of clot formation. There should be adequate light while observing for clot formation. Wipe the base of the tube to remove additional water as it hampers reading of the test results
9. Incorrect addition of reagent	The test requires extreme precision, besides the addition of the reagents should be as per the instructions given in the package insert. Exactly 0.1 mL of plasma, 0.1 mL of Liquicelin-E reagent and 0.1 mL of calcium chloride prewarmed at 37°C should be used. Well-calibrated micropipettes should be used for dispensing
10. Temperature of water bath not set correctly	The temperature of the water bath should be set at 37°C ± 1°C
11. Cotaminated reagents (APTT and calcium chloride) and glassware	Check the working of the reagents with normal control plasmas. Check the reagents for turbidity. Ensure that clean and dry glassware and micropipettes are used
12. Clotting times of patients on anticoagulant therapy depends on the type and dosage of anti-coagulants and also on the time lag between specimen collection and the last dose of anti-coagulant	The history of the patient must be taken carefully to determine the anticoagulant used as well as the time lag between the specimen collection and the last dose of anticoagulant
13. Improper storage condition leading to reagent precipitation	To maintain the sensitivity and performance of the reagent, avoid thermal stress due to exposure to high ambient temperature thereby causing precipitation, ensure that the reagent is stored at the recommended temperature

Problem: Shortened Clotting Time

Possible causes	*Solutions*
1. Broken glassware allowing silica to trigger the clotting reaction	Ensure that broken glassware is not used for testing purposes
2. Administration of drugs/other clinical conditions	APTT tests are shortened because of the administration of drugs, oral contraceptives or conjugated estrogen therapy. It is therefore essential to know the patient history to determine the type of anticoagulant used and the time lag between the last dose of the anticoagulant and specimen collection

FIBRINOGEN DEGRADATION PRODUCTS (D-DIMER) ESTIMATION

Problem: False-positive Results

Possible causes	*Solutions*
1. Markedly lipemic, hemolyzed and contaminated serum samples could produce nonspecific results	Avoid using lipemic, hemolyzed and contaminated samples for testing
2. Drying of reagent on the slide	Do not read results beyond 3 minutes. The test should not be carried out directly under the fan
3. Presence of dust or debris on the glass slide used	Dust or debris could be misinterpreted as agglutination therefore only clean and dry glass slides must be used for testing
4. Latex particles contaminated with positive control/positive sample	Care must be taken to see that the reagent dropper tip does not touch the sample taken on the slide during dispensing of the reagent
5. Dried latex particles observed in the latex reagent ▪ During slide test with negative control ▪ In the dropper of the vial (due to freezing of the latex reagent during storage) ▪ Improper dispensing of the entire reagent from dropper	Immediately after performing the test, transfer the contents of the reagent dropper back into the reagent vial. Ensure that no reagent is left behind in the dropper. Close the cap of the reagent vial properly and store it back at 2–8°C. Do not freeze the reagent vial
6. Activation of coagulation system with subsequent microvascular fibrin deposition and lysis has been reported in diverse clinical conditions such as trauma, surgery, inflammation and malignancy	Elevated levels of plasma XL FDP may be expected to occur in such conditions. Note the clinical history of the patient before arriving at a final diagnosis

Problem: Delayed Agglutination

Possible causes	**Solutions**
1. Cold reagents are used for testing	Bring all reagents and samples to room temperature before commencing the testing procedure

Problem: False-negative Results

Possible causes	Solutions
1. Serum is used as test specimen	Always use plasma as test specimen for performing the test
2. The reagent may be damaged due to microbial contamination or exposure to extreme temperatures	Check the performance of the latex reagent using known positive samples, if the latex reagent is working then the positive control may have deteriorated
3. Weak agglutination is difficult to interpret	Shake the latex reagent well before use to disperse the latex particles uniformly and improve the test readability
4. Excess sample dispensed leading to prozoning	Dilute the plasma and check for agglutination. If no agglutination is observed with the neat sample but agglutination is observed with the diluted sample, then it may be due to prozoning. Determine the titer. Dispense exact amount of sample as mentioned in the package insert
5. Samples stored for a long period of time are used as specimens	Fresh samples are preferable for testing; however, samples can be stored for up to a week at 2–8°C
6. In PE, if the clots are of small size. The amount of D dimmer released from very small clots may be diluted by the circulation	
7. Patients with stabilized clots and not undergoing active fibrin desposition and plasmin activation may not give detectable D dimmer elevations	The clinical history of the patient must be taken consideration to determine the D dimmer levels in the patient
8. In case of hereditary, acquired deficiency and dysfunction of fibrinogen	
9. If the conclusion of false at by comparison with another kit, then this other kit could be giving a false-positive reaction	Run the test with a third kit to validate results. negative result has been arrived

Problem: Positive Control Giving Negative Reaction

Possible causes	*Solutions*
1. The positive control may have deteriorated due to contamination or exposure to extreme temperatures	Check the performance of the latex reagent; using known positive samples, if the latex reagent is working then the positive control may have deteriorated

CHAPTER

25

Immunohematology Blood Banking

The ABO and Rh are the major (clinically significant) blood group antigens though almost 400 of them have been recognized. Given below are the more important blood group systems:

1. ABO, 2. Rhesus, 3. Dell, 4. Duffy, 5. Kidd, 6. Lutheran, 7. Lewis, 8. P 9. MNS, 10. I. (All of the above if mismatched can cause hemolytic transfusion reaction and 1–6 can be responsible for hemolytic disease of the newborn).

BLOOD GROUP ANTIBODIES

NATURALLY OCCURRING ANTIBODIES

Under normal circumstances, the newborn has no ABO antibodies. However, after 10–20 weeks later, moderate amount of antibodies are present which appear without any specific antigenic stimulus. So, these are called naturally occurring antibodies. Anti-A and anti-B are important examples of this class. They are IgM immunoglobulins; they react optimally at room temperature, they are also called cold antibodies. They are complete antibodies in serological behaviors, because these antibodies readily agglutinate the red cells carrying the corresponding antigen in saline.

Immune antibodies are produced in response to immunization either by transfusion or pregnancy. They are usually IgG antibodies; they react best at body temperature 37°C, and are called warm antibodies. Rh antibody (anti-D) is important immune antibody. They are often called incomplete antibodies, as they do not cause agglutination of red cells with corresponding antigen in saline. These antibodies cause only sensitization or coating of the red cells.

In 1900, Karl Landsteiner discovered the blood groups ABO and classified blood into A, B and O groups. A fourth blood group 'AB' was discovered by Landsteiner's associates, von Decastello and Struli in 1902.

The four blood groups are determined by the presence or absence of blood group antigens (agglutinogens) on red blood cells and accordingly an individual's group is A, B, AB or O (O denotes absence of A or B antigens). In addition, it has been shown that corresponding to antigen A and B, there are naturally occurring antibodies anti-A and anti-B (agglutinins) in the plasma/serum of individuals whose red cells lack the corresponding antigen. Group A individuals have anti-B, group B individuals have anti-A, group O individuals have both anti-A and anti-B and group AB individuals have no agglutinins in plasma/serum (Table 25.1).

Table 25.1: ABO antigens and corresponding antibodies		
Antigen on RBC	*Antibody in plasma/serum*	*Blood group*
A	Anti-B	A
B	Anti-A	B
AB	None	AB
None	Anti-A and anti-B	O

It was further shown that group A could be subdivided into two principal subgroups A_1 and A_2. On the basis of this, ABO system is divided in six main groups A_1, A_2, B, A_1B, A_2B and O (Table 25.2).

Table 25.2: Possible phenotypes and genotypes in ABO group system	
Phenotypes	*Genotypes*
A_1	A_1A_1, A_1, A_2–A_1, O
A_2	A_2A_2, A_2O
B	BB, BO
A_1B	A_1B
A_2B	A_2B
O	OO

GENETICS OF ABO SYSTEM

ABO system follows Mendelian law of inheritance. The locus for ABO grouping is a chromosome 9 which is occupied by one of three major allelic genes namely A, B and O. Each individual has a pair of chromosomes (one from each parent). The A and B genes are dominant, while O gene is recessive, thus, not detected directly and accordingly absence of A and B antigens on red cells indicates 'O' blood group (Fig. 25.1).

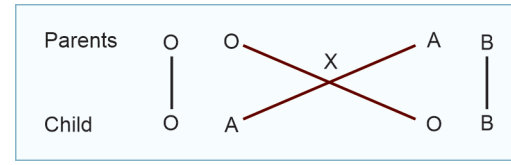


Fig. 25.1: The possible phenotypes and genotypes in ABO group system

BIOCHEMISTRY

The expression of A and B genes appears to be dependent on another gene called H gene. H gene is inherited, independent of A, B and O genes. H gene is expressed both as homozygous (HH) and heterozygous (Hh). When no H gene is inherited, a (hh) phenotype results which is extremely rare. This is commonly called Bombay group. Bombay group individuals are homozygous for hh gene.

As shown in Fig. 25.2 there is a basic precursor substance which is converted to H substance by an enzyme L. Fucosyl transferase (a product of H gene).

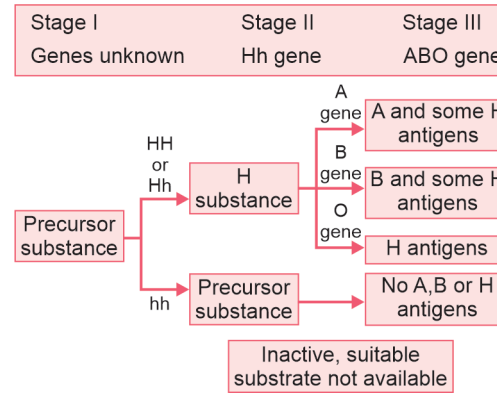


Fig. 25.2: Stages in production of blood group determinants of red cells

H substance is next acted upon by A and/or B gene specified transferase enzyme and is converted to A and/ or B antigens. The 'O' gene is an amorph (no gene product) and thus, group 'O' cells enter only H substance.

The persons with genetic configuration of 'hh' cannot act on the precursor substance and the precursor substance remains unaltered. Since no H substance is produced, the ABO genes remain inactive and there is no conversion to A, B or H antigen (These are the persons called Bombay group).

SUBGROUPS OF A AND AB

Anti-A serum very seldom differentiates between A_1 and A_2. For this reason we use human anti-A_1 and lectin anti-A which agglutinate A_1 and A_1B cells but not A_2 and A_2B cells. About 20% of persons with A antigen in A or AB group are A_2 or A_2B.

It is not necessary to classify group A patient or donors as A_1 or A_2 except when the individual serum contains anti-A_1. Anti-A_1 occurs in the serum of 1–8% of A_2 group persons and 22–35% of A_2B group persons.

Anti-A_1 causes discrepancies between ABO cell and serum groupings and may cause crossmatch incompatibilities, but it is considered clinically significant if it reacts at 37°C.

Subgroups weaker than A_2 occur infrequently. They are characterized by declining number of 'A' antigens on red cells and reciprocal increase in H reactivity. Weaker variants of A are A_3, Ax, Am and A intermediate.

Subgroups of B

Subgroups of B are clinically not significant, but they can be Bx and Bm type.

Antibodies of ABO System

The antibodies in ABO are usually naturally occurring and are mostly IgM. However, IgG classes are also present. IgG anti-A and IgG anti-B are found more commonly in group O individuals.

Anti-A and anti-B are usually not produced in infants up to age of 3–6 months. However, they reach a maximum titer by 5–10 years and then gradually become weaker as the individual ages. The antibodies found in the serum of infants at birth are almost of maternal origin. The serum grouping of a newborn is, therefore, not recommended.

Anti-H

Anti-H very rarely occurs as cold reactive agglutinin in individuals with very low levels of H antigens on their cells and have little clinical significance. However, anti-H found in Bombay blood group (Oh) is an all antibody and is clinically significant. It occurs as a hemolysin and agglutinates cells at 37°C.

ABO TESTING PROCEDURES

- For demonstration of true ABO group of an individual, it is important to do both cell grouping (forward typing) and serum grouping (reverse typing). Both forward and reverse typing must match to confirm the true ABO type of an individual.
- Serum should always be added before adding the cells and examine each tube after serum has been added to ensure that none has been left.
- ABO grouping test should be done at room temperature, or below. Testing at 37°C weakens reaction.
- Tubes, slides and microplates should be labeled properly.

ABO GROUPING

SLIDE ABO GROUPING TEST

1. Prepare a 10% suspension in saline of the red cells to be tested.
2. Mark the left slide of a clean glass slide 'anti-A' and the right side 'anti-B'.
3. Add to the left side a drop of anti-A grouping serum and to the right side a drop of anti-B grouping serum.
4. Next to the antisera drops, place 1 drop of 10% saline suspension of unknown red cells. The contents on the left and right sides should not get mixed up.
5. With one-half of an applicator stick, mix the red cell suspension with the anti-A serum; and with the other half-mix the red cell suspension with the anti-B serum.
6. Gently rock the slide back and forth and observe the mixture for one minute unless agglutination occurs earlier.
7. It is necessary to confirm the slide grouping by testing the unknown serum with the known red cell suspensions of groups A, B, and O as indicated for six-tube method. (In stead of 10% saline suspension, citrated/oxalated blood or direct blood from skin puncture may be taken. Slow and weak agglutinations may occur in A subgroups).

SIX-TUBE METHOD (Fig. 25.3)

1. Separate the serum from red cells or clot of unknown specimen.
2. Prepare a two per cent suspension in saline of the red cells of the unknown specimen.
3. For each specimen, six test tubes are labeled showing in addition to proper identification of the unknown specimen the under-mentioned information: Tube 1, anti-A; tube 2, anti-B; tube 3, anti-AB; tube 4, A; tube 5, B; tube 6, O.
4. Place 1 drop of anti-A serum in tube 1, 1 drop of the anti-B serum into tube 2, and 1 drop of the anti-A, B serum into tube 3.
5. Add two drops each of the serum of the unknown specimen to tubes 4, 5, and 6.
6. Add one drop each of the 2% red cell suspension in saline of the unknown specimen to tubes 1, 2, and 3.
7. To tube 4, add 1 drop of a 2% red cell suspension in saline of group A cells; to tube 5, add 1 drop of a 2% red cell suspension in saline of group B cells; and to tube 6, add 1 drop of a 2% suspension of red cells in saline of group O cells.
8. Mix the contents of all tubes by shaking the test tube rack.

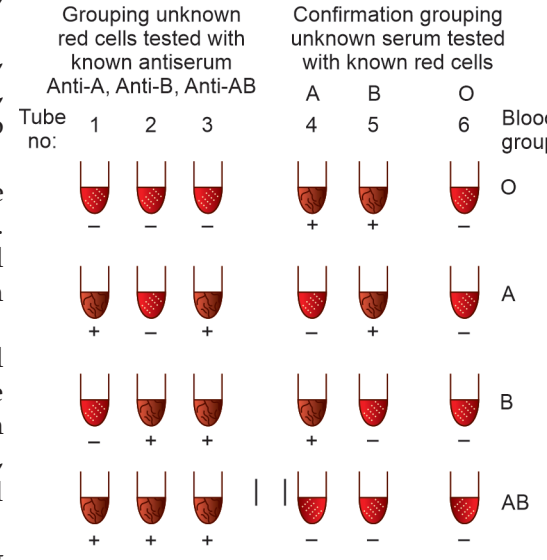


Fig. 25.3: ABO blood grouping—tube method

9. Depending on the speed necessary for completing the test, one of the two alternatives may be chosen: (i) the test tubes may be left at room temperature for at least 2 hours, or (ii) after 2–3 minutes, the tubes may be centrifuged (1 minute at 1500 rpm in a clinical centrifuge).
10. After incubation or centrifugation, the red cell suspensions are redispersed by tapping the tubes. Presence of agglutination is checked with the naked eye, with the scanning lens of a microscope and if the results are doubtful or negative, microscopically (10x objective) after placing a drop on a slide.

Interpretation

The results of an ABO grouping test can be accepted as valid only if the findings obtained in the first three test tubes with known antisera agree with those obtained in the second three test tubes with known red cell suspensions; this second part of the test is called confirmation, check or reverse grouping. Given on previous page are the result in the four main ABO groups in such tests when both the unknown red cells and unknown serum are tested. Agglutination is indicated by the + sign, – sign indicates no agglutination.

Absent or weak agglutination with the unknown serum may be simulated by hemolysis of the known red cells, this suggests the presence of hemolytic anti-A or anti-B antibody. The assumption that hemolysis is due to anti-A or anti-B must be confirmed by repeating the test with the serum after its inactivation in a water bath at 56°C for 30 minutes—use of the inactivated serum is expected to result in agglutination instead of hemolysis.

If discrepancies in the two parts of ABO grouping are observed, the test should be redone. If a second test reveals the same discrepancy, the following possibilities are to be considered:

1. Cold agglutinins may cause agglutination of the known cells by the unknown serum regardless of its ABO group. Confirm this by testing the unknown serum with its own red cells at 4°C for 1–2 hours. Under those conditions cold agglutinins produce agglutination, which disappears after transfer of the specimen to a water bath at 37°C for 5–10 minutes.
2. Agglutination of the unknown red cells by known antisera that conflict with the results obtained in the confirmation grouping may be due to coating of the red cells by autoantibodies. Confirmation of this phenomenon is obtained by performing on the unknown red cells the direct antiglobulin test and obtaining positive results. In order to obtain in such cases a reliable ABO grouping, the red cells should be washed several times with large amounts of isotonic saline solution, following which they are more likely to give adequate result.
3. Unexpected agglutination obtained with the unknown serum may reflect presence of irregular agglutinins, such as Rh antibodies, which react with the corresponding blood factor in the suspensions of the known red cells.
4. Discrepancies in the two parts may some-times be present due to A subgroup. In which anti-A_1 serum, anti-A_2 serum, and A_1 and A_2 reagent red cells are used for confirmation.
5. Serum of newborn and young infants may not contain the isoagglutinins expected from the reactivity of their red cells; hence, in infants, the use of unknown serum is not a reliable method. Much less frequently isoagglutinins may be absent in older children and adults due to hypo or agammaglobulinemia or for unknown reasons.

Briefly the reasons can be described as:

a. Improper identification of specimen.
b. Improper techniques like:
   - Failure to add proper reagent
   - Incorrect cell to serum ratio
   - Failure to identify hemolysis
   - Incorrect reading, recording or interpretation of test results.
c. Failure of equipment
d. Poor standardized or stored reagent

e. Patients problems
   - Patient may fail to express ABO antigens on red cells, e.g.
     1. Age (newborn or old age)
     2. Disease states, i.e. leukemia or lymphomas.

This leads to false-negative results

Acquired B-antigen can occur
1. Gram-negative septicemia
2. Carcinoma colon

This may cause false-positive reaction

- Rouleaux formation: This is an aggregation of red cells in the form of piles of coins and can be misinterpreted as agglutination.
- Acquired antibodies, e.g. anti-A_1, in A_2 persons anti-H in Bombay phenotype, cold autoantibodies, all unexpected antibodies
- Absence or weakening of antibodies, e.g. immune deficiency states. Agammaglobulinaemias, etc.

Solving Problems of Discrepancies

Once a discrepancy is detected in ABO cells and serum grouping, repeat the test before additional investigations are carried out. Quality assurance of reagent, correct technique, careful observation and interpretation of results resolve many problems.

Repeat Preliminary Procedures

1. Obtain a fresh blood sample from donor unit or patient to rule out discrepancy due to contamination or unidentified samples.
2. Wash the cells 3–4 times in normal saline to rule out rouleaux formation and prepare 2–5% cell suspension.
3. Perform direct antiglobulin test on the cells, to detect if cells are coated with antibody as in HDN and AIHA.
4. Retest the cells with fresh and potent anti A, anti-B, anti-AB, anti-A or anti-H as appropriate for individual problem.
5. Test the serum against appropriate A_1, A_2 and B cells. Group O cells and autologous cells should be used as controls to detect alloagglutinins and autoagglutinins.
6. Use group O cord cells if anti-I is suspected.

RH BLOOD GROUP SYSTEM

It had been suspected for a long time that cause of many transfusion reactions was due to specific differences in blood other than the four main blood groups originally described by Landsteiner. In 1939, Levine and Stetson described an antibody in the serum of a group O mother who delivered stillborn fetus and subsequently developed symptoms of hemolytic transfusion reaction when transfused with her husband's group O blood. They noted that the responsible antibody developed in the mother through an antigenic factor from the fetus. The antibody was not named at that time.

In 1940, Landsteiner and Wiener immunized rabbits and guinea pigs with red cells of rhesus monkeys. The serum of immunized rabbits contained an antibody named anti-Rh which agglutinated red cells in approximately 85% of white population tested. Its antigenic determinant was called Rh factor. The antibody discovered by Levine and Stetson in

the mother was subsequently re-examined and found identical in activity as the anti-Rh antibody of Landsteiner and Wiener. This work led to the discovery of Rh system.

Clinical Importance of Rh

Rh blood group system is important because of:

1. Hemolytic disease of newborn (HDN) may occur in Rh negative pregnant women with Rh positive fetus.
2. Rh antibodies may develop in Rh negative patient if given Rh positive blood.

Present Status and Nomenclature

The genes of the Rh system are present on chromosome 1. There are three pairs of genes called Cc, Dd, and Ee but only five antigens (C, c, D, E, e) as there is no antigen produced by the 'd' gene. Rh gene travels in the set of three, e.g. CDC, CDE, cDE, etc. with one set being received from each parent. There are thus, eight possible chromosomes each of which carries genes for three factors (Table 25.3).

Table 25.3: Eight basic chromosomes of Fisher and Race

DCe (R_1)	dce (r)
DcE (R_2)	dCe (r′)
Dce (R_0)	dcE (r″)
DCE (R_z)	dCE (r^y)

Any two chromosomes, one from each parent may be inherited by an individual so that 36 different genotypes are possible Table 25.4 gives the most common genotypes.

Out of various gene combinations as shown in Table 25.4, it is presence or absence of D gene which is most important. When a person inherits 'D' antigen its red cells react with anti-D and these are called Rh-D positive. If a person does not inherit D antigen, the red cells do not react with anti-D and thus, called Rh-D negative.

Table 25.4: Common genotypes

DCe/dce	(R_1r) dce/dce (rr)
DCe/DCe	(R_1R_1)
DCe/DcE	(R_1R_2)
DcE/dce	(R_2r) dcE/dce (r″r)
DCe/DCe	(R_1R_0)
DcE/DcE	(R_2R_2)
Dce/dce	(R_0r) ,
DCe/dcE	(R_1r'')
DCe/dCe	(R_1r') dCe/dce (r′r)

Rh D^u Antigen

It is defined as weakened expression of the normal D antigen, i.e. there are fewer than normal D antigens per red cells. There are two grades of D^u:

- High grade D^u
- Low grade D^u

High grade D^u red cells are agglutinated by certain anti-D sera while low grade D^u are mostly detected by (antihuman globulin) AHG test.

It has been shown that D^u positive bloods may immunize the Rh negative patient resulting in the formation of anti-D antibodies, hence, it is important to exclude D^u individuals from Rh negative blood donor list.

Rh Antibodies

Rh antibodies are usually immune IgG type but may be IgM or even IgA. Most Rh antibodies result from exposure to Rh positive red cells, either due to pregnancy or transfusion in individuals who lack the corresponding antigen.

Auto-Rh antibodies may be found in individual with warm autoimmune hemolylic anemia and anti-e is the most commonly found antibody.

Reagents for Rh(D) Grouping

Both polyclonal and monoclonal reagents are available in different combinations.

I. Polyclonal human and D serum
   a. Anti-Rh(D) serum for saline or rapid tube test (high protein medium).
      This contains macromolecular additives and gives rapid reliable results.
   b. Anti-D for saline tube test
      1. Anti-D-IgM
      2. Anti-D-IgG

II. Monoclonal Anti-D Reagents
   1. IgM anti-D monoclonal Reagent
   2. IgG anti-D monoclonal Reagent
   3. IgM and IgG (Blend) anti-D monoclonal reagent
   4. Blend of IgM monoclonal and IgG polyclonal reagent.

The IgM anti-D monoclonal is highly specific, saline reacting working well at room temperature and at 37°C. It is good for emergency slide test, or immediate spin tube test as well as routine Rh-D typing, but IgM anti-D are unreliable for detecting weak D by antiglobulin test, while IgM and IgG (Blend) monoclonal reagent or IgM anti-D monoclonal and IgG anti-D (polyclonal) blend can be used for weak D testing by antiglobulin test.

RH(D) GROUPING PROCEDURES

Rh(D) grouping is done along with ABO grouping using same techniques as used for ABO grouping:

1. Slide or tile method
2. Tube method
3. Microplate method.

Slide Testing

Slide test is not recommended for routine test because it is not reliable especially for weak reactive cells.

Method

1. Place one drop of anti-Rh (D) on a labeled slide.
2. Place one drop of 22% albumin on another labelled slide to serve as control.
3. Put one drop of 40–50% red cells on both the slides.
4. Mix the cell suspension and reagent, using a clean stick for each slide and spread the mixture evenly on the slide over area of 15 mm diameter.

5. Place both slides on a view box (lighted), till gently and continuously for two minutes. Observe for agglutination.

A positive test has agglutination with anti- Rh(D) in the 'test' and a smooth suspension of cells in control. A negative test has smooth suspension of cells in both 'test' and 'control.

Tube Method

1. Place one drop of anti-Rh(D) serum in a tube labeled test
2. Place one drop of 22% albumin in a tube labeled 'control'.
3. Add 1 drop of 2–5% cell suspension in plasma or serum in each tube.
4. Mix well and keep at 37°C for 1 hour (sedimentation method).
   In case of emergency, incubate the tube for 10 minutes at 37°C and then centrifuge at 1000 rpm for 1 min (spin tube).
5. Gently resuspend the cell button and observe for agglutination. All negative results must be confirmed under microscope.

Interpretation

A positive test has agglutination with anti-Rh(D) in the test and a smooth suspension of cells in the control. A negative test has smooth suspension of cells in both 'test' and 'control'.

Testing for Du

Method

1. Take one drop of anti-Rh(D) serum in a clean labeled test tube.
2. Place one drop of appropriate control in a labeled tube.
3. Add 1 drop of 2–5% cell suspension to be tested to both the tubes.
4. Mix and incubate both the tubes at 37°C for 15–30 minutes.
5. Centrifuge at 1000 rpm for 1 minute.
6. Gently resuspend the cell button and examine for agglutination. If there is strong agglutination of cell in 'test' tube, then sample is Rh(D) positive and there is no need to proceed with antiglobulin phase of test.
7. If no agglutination or doubtful reaction is observed, wash the cells 3–4 times with saline and decant the last washing.
8. Add 1–2 drops of antiglobulin reagent (Coombs), mix gently and centrifuge at 1000 rprn for 1 minute.
9. Resuspend the cell button gently and examine for agglutination and record the results.
10. If the test is negative, the reaction can be confirmed by adding known IgG sensitized cells, recentrifuge and reexamine for agglutination. Presence of agglutination confirms the test result.

Interpretation

Agglutination in the 'test' tube and none in negative control tube constitutes a positive test result and blood is accordingly labeled Rh-Du positive.

ANTIHUMAN GLOBULIN TEST

Historical Perspective

The first step towards a rational approach to blood transfusion became possible with Landsteiners discovery in 1901 of the ABO blood group system. Nearly half a century

later, the next advance occurred with the introduction of serological techniques permitting the detection of incomplete antibodies, which are nonagglutinating immunoglobulin G (IgG) antibodies that sensitize red blood cells. In 1945, Coombs et al. described the use of antihuman globulin test for the detection of weak and non-agglutinating antibodies in serum. Further in 1946, Coombs et al. described the use of antihuman globulin test to detect in vivo sensitization of red blood cells in babies suffering from hemolytic disease of the newborn. Although the test initially was of great value in investigating Rho (D) hemolytic disease of the newborn, it was not long before its versatility for the detection of other IgG blood group antibodies became evident.

Although Coombs et al. were instrumental in introducing the antihuman globulin test to blood group serology, the principle of the test had, in fact, been described by Moreschi in 1908. Moreschi studies involved the use of rabbit antigoat antiserum to agglutinate rabbit red blood cells that were sensitized with low non-agglutinating dose of goat anti-rabbit red cell serum.

Later, in 1957, Dacie et al. published data regarding the importance of antihuman complement activity. Further, in 1960, many reports were published indicating the need for antihuman complement activity in antihuman globulin serum to allow the detection of antibodies in the indirect test. Though many of the antibody specificities mentioned in these reports are now considered of little clinical significance; however, one specificity that was consistently mentioned and is still considered to be clinically significant was Anti-Jka. Further it was also shown that the presence of antihuman complement activity would enhance the reaction of some antibodies namely anti-Fy^a and anti-K.

PRELIMINARY NOTES: ANTIGEN AND ANTIBODY RELATED TO BLOOD GROUP SEROLOGY

Antigens are foreign substances, which initiate an immune response in human body and react specifically with antibodies. Blood group antigens that are located on the surface of red blood cells when transferred to another individual are almost certain to carry antigens not possessed by the recipient. These foreign antigens then initiate an immune response and production of antibodies *in vivo*. Antibodies are counter agents produced as a defence mechanism when foreign antigen enters human body. With respect to blood group serology, it means that blood group antibodies are specific and are formed against red blood cell antigens not possessed by the recipient.

ANTIBODY NOMENCLATURE WITH RESPECT TO BLOOD GROUP SEROLOGY

Red blood cell antibodies may be naturally occurring or immune in nature. Naturally occurring antibodies are produced because of the exposure of human body to certain bacteria and plant substances whose antigenic structure resembles blood group antigens. Naturally occurring antibodies are most often IgM class of antibodies.

Immune antibodies (alloantibodies) are antibodies present in an individual due to transfusion or pregnancy. Immune antibodies most often are IgG class of antibodies, sometimes IgM or a mixture of IgM and IgG class of antibodies.

IGM CLASS ANTIBODIES

IgM antibodies are pentamers in which five polypeptide subunits are held together by disulphide bonds and joined by J chain. IgM antibodies do not cross the placenta but are most effective in activating complement (Fig. 25.4).

Sialic acid present on the red cell membranes impart to the cells, a net negative charge. As a result mutual repulsive force exists between red blood cells known as zeta potential

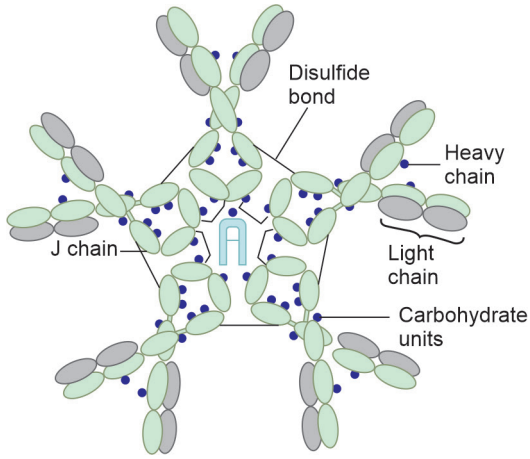


Fig. 25.4: Illustration of IgM molecule

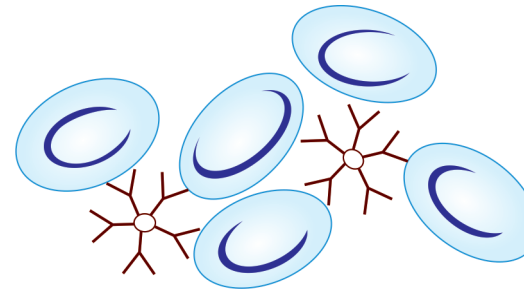


Fig. 25.5: Illustration of complete antibodies

and the red blood cells are separated by a distance. In blood group serology, IgM antibodies are termed as complete antibodies because of their ability to directly bridge the distance between two red blood cells in spite of the zeta potential and bring about visible agglutination. IgM antibodies are able to bridge this distance and bring about visible agglutination (Fig. 25.5).

IgG CLASS ANTIBODIES

IgG antibodies are monomers with two heavy chains and two light chains. IgG is the only immunoglobulin that can cross the placental barrier, a fact which explains the role of IgG antibodies in hemolytic disease of the newborn. Four subclasses of IgG are present and they vary widely in their ability to activate complement (Fig. 25.6). IgG3 molecules are highly effective in activating complement, IgG1 moderately activate complement, IgG2 slightly activate complement and IgG4 do not activate complement.

IgG antibodies are also known as incomplete antibodies because they cannot bridge the distance between two red blood cells and bring about direct agglutination like the IgM immunoglobulins. Antibodies that do not agglutinate red blood cells directly are termed as incomplete antibodies or sensitizing antibodies and the red blood cells that have such antibodies on their surface are known as sensitized cells. Incomplete antibodies sensitized onto red blood cells can be agglutinated by antihuman globulin reagent through the usage of enhancement medium to visualize agglutination (Figs 25.7 and 25.8).

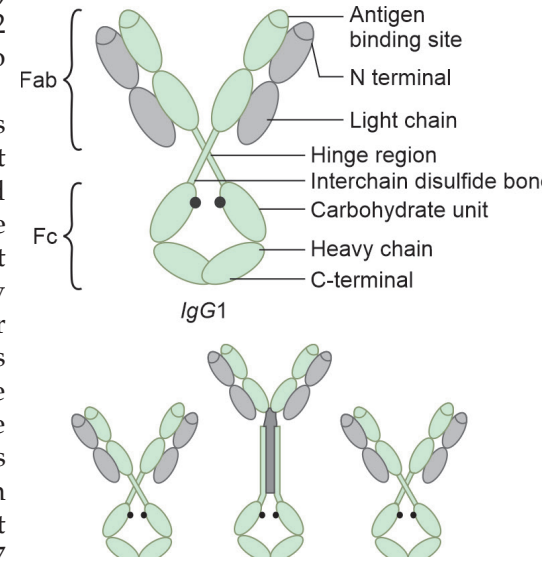


Fig. 25.6: Structure of four subclasses of IgG

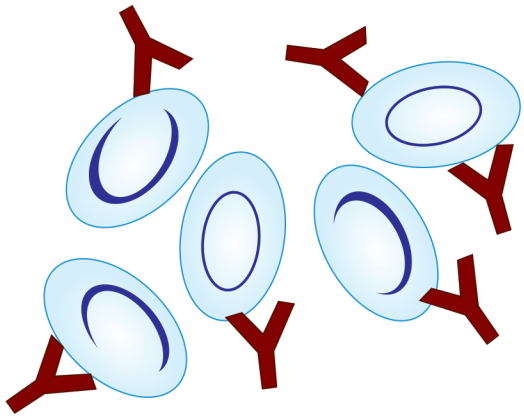


Fig. 25.7: Illustration of red blood cells sensitized with incomplete antibodies

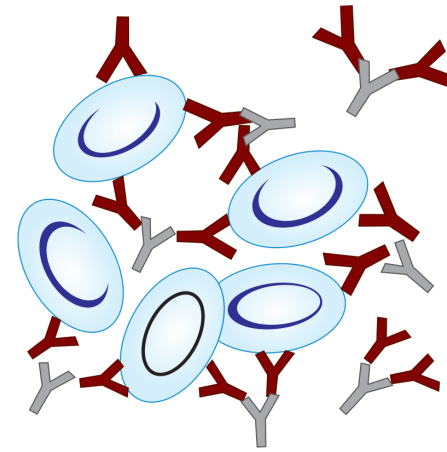


Fig. 25.8: Illustration of IgG sensitized red blood cells agglutinated by AHG reagent

ROLE OF ENHANCEMENT MEDIUM

An enhancement medium can either be a protein (e.g. bovine serum albumin) or enzymes (e.g. papain, bromelin, ficin) or low ionic salt solution (LISS) or charged molecules (polybrene) that reduce zeta potential, enhance antibody uptake, enhancement in agglutination reactions (Fig. 25.9).

In order to clearly understand antibody enhancement methods, it is important to consider the basic aspect of haemagglutination reactions. Agglutination is a reversible chemical reaction and occurs probably in two stages:

Stage 1: Sensitization, i.e. attachment of antibody to red blood cell membrane antigen.

Stage 2: Formation of bridges between the sensitized red blood cells to form lattice (cross-linking) to visualize agglutination.

Thus, various factors that affect these two stages can be manipulated to enhance agglutination.

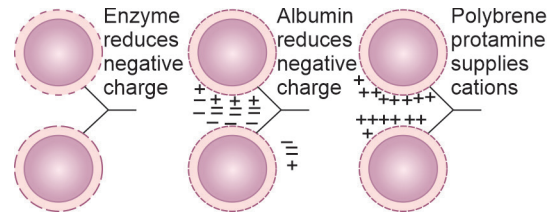


Fig. 25.9: Illustration of antibody enhancement

ENHANCEMENT TECHNIQUES

Albumin Additives

Bovine serum albumin (BSA), a dipolar protein has been used routinely for many years as an enhancement medium. The enhancement effect of albumin is attributed to its influence on the second stage of agglutination by reducing the net negative charge on the red blood cell membranes thereby potentiating agglutination. Bovine serum albumin is available as solutions of 22% or 30% concentration.

Enzymes

Treating red blood cells with certain enzymes enhances agglutination of antibodies with red blood cell antigens. Most commonly used enzymes in immunohematological procedures are papain, bromelain and ficin which belong to thiol protease class of enzymes.

Proteolytic enzymes reduce the red blood cell surface charge by cleaving sialic acid molecules on red blood cell membrane, lowering the zeta potential and enhance agglutination. Enzyme treatment also causes spicule formation on the red blood cell membrane. This greatly increases the potential number of contact points for antibodies to react with corresponding antigens on red blood cell membrane.

The reaction of anti-P, Lewis and Kidd blood group antibodies are stronger with enzyme treated red blood cells. Another important observation is that Lewis and Kidd blood group antibodies may lyse enzymetreated red blood cells.

Positively Charged Molecules

In the presence of positively charged polymers, such as hexadimethrine bromide (Polybrene®), protamine sulfate and poly-L-lysine, normal red blood cells exhibit spontaneous aggregation. Thus, any red blood cells sensitized with antibody will exhibit enhanced agglutination.

The action of these charged molecules may be due to neutralization of negative charge contributed by sialic acid and release of water molecules that cover red blood cell membrane thereby enhancing agglutination.

Polybrene® generally is added to red blood cells that have been incubated with antibody at low ionic strength and low pH.

Polyethylene Glycol (PEG)

PEG is a water-soluble polymer used as an additive to increase antibody uptake. Its action is to remove water molecule that takes up more space around the red blood cell membrane, thereby enhancing antibody uptake and reaction strength.

Low Ionic Salt Solution (LISS) and LISS Additives

LISS (approximately 0.03 M) greatly increases stage 1 uptake of antibody to red blood cells, as compared to normal saline (approximately 0.17 M). To prevent lysis of red blood cells at such a low ionic strength, a non-ionic substance, such as glycine, is incorporated in LISS.

LISS additives may sometimes contain macromolecules (e.g. BSA) in addition to ionic salts and buffers. Addition of macromolecules to LISS reduces ionic strength with shortened incubation times.

COMPLEMENT

An observable phenomenon that may result from red blood cell antigen–antibody interaction is hemolysis. This requires cooperation of another defense mechanism of body known as the complement system. Complement is the name given to a set of approximately twenty serum proteins, which in response to antigenic stimulus react with one another in a sequential cascade.

The most important complement component is C3b, which can be activated either by the classical (Fig. 25.10) or the alternate pathway. The antigen-antibody

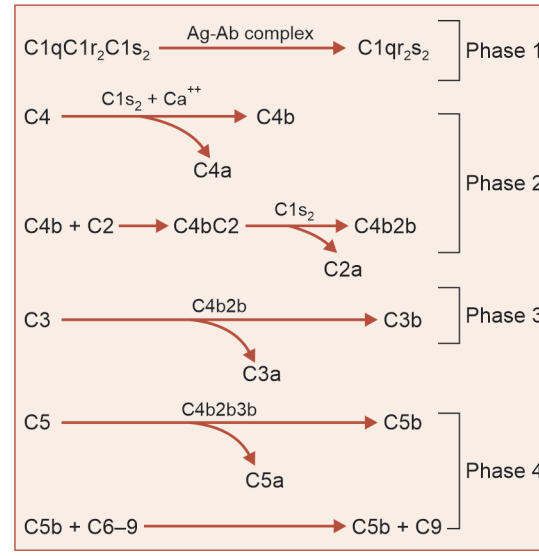


Fig. 25.10: Activation of classical complement pathway

interactions activate the classical pathway that is of great significance in blood group serology.

Immunoglobulin Requirements for Activation of Classical Pathway

The mode of activation of classical pathway is through binding of C1q to CH_2 domain of IgM or to the CH_2 domain of IgG1 or IgG3 antibodies.

The C1q molecule has six Fc-binding sites and in order to make a firm bond with antibody molecule, at least two of these sites must bind to antibody (Fig. 25.11).

In case of IgM antibodies, when the molecule is in planar or star form, there is only a single binding site for C1q, as a result it cannot bind firmly and activate C1q. However, when combined with antigen, IgM molecule usually assumes staple form with the F(ab) at right angle with Fc. The distortion produced by this movement exposes an additional C1q binding site. As a result IgM antibody combined with its corresponding antigen bind to two C1q sites on single IgM molecule (Fig. 25.12).

In case of IgG antibodies, two IgG antibodies must be present on antigen surface at a distance of 20–30 nm apart from each other. This is the maximum span of the C1q molecule. It is for this reason IgM is considered more effective in activating C1q than IgG molecule.

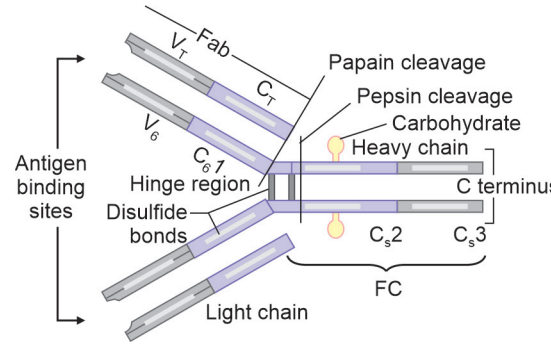


Fig. 25.11: Illustration of immunoglobulin molecule

An important point to note is that all examples of immune anti-D fail to activate complement. The reason being that the D antigenic sites are too far apart from each other on the red blood cell surface. As discussed, two IgG molecules must be at a distance of 20–30 nm apart from each other so as to activate C1q. Since D antigenic sites are too far apart from each other, the bound IgG molecules cannot activate C1q.

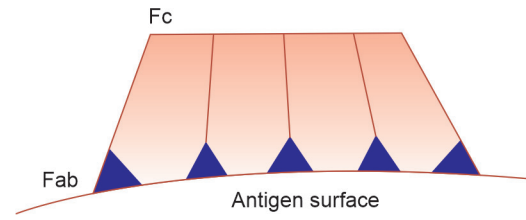


Fig. 25.12: Illustration of staple form—IgM molecule

Complement-binding Antibodies (Table 25.5)

Complement components may attach to red blood cells *in vivo* or *in vitro* via one of the probable mechanisms.

- Complement-binding alloantibody (blood group antibodies present in an individual due to transfusion or pregnancy) specific for red blood cell antigen may cause sensitization of complement component on red blood cell surface due to transfusion.

  Complement-binding alloantibodies when present in low concentrations bring about the attachment of complement component up to the C3 stage, but produce little or no hemolysis. Further activation of C3 occurs only when complement is powerfully activated due to high concentration of antibodies, leading to the generation of large amount of C3b. Certain antibodies such as Anti-Jk^a are seldom lytic but invariably activate complement thereby leading to the deposition of complement components on the red blood cell surface.

Table 25.5: Complement binding by human red cell antibodies

System	*Antibody*	*Readily detectable hemolysis*	*Positive antiglobulin test (anticomplement)*
ABO	Anti-A		
	Anti-B	Many	Many
	Anti-A, B		
	Anti-A^-	None	Very few
	Anti-HI	None	Few
	Anti-H^t	All	All
Lewis	Anti-Le^a	Some	All
	Anti-Le^b	Few	Many
P^-	Anti-P_1	Few	All
	Anti-PP_1 P^k	Few	All
	Anti-P (auto-)	All	All
	Anti-P (allo-)	Many	All
Li	Anti-l auto-Anti-1	Many	Many
Sd	Anti-Sda	None	Some
Rh	Anti-D	None	None
	Anti-c, E, etc.	None	None
K	Anti-K	None	Many
Fy	Anti-Fy^a	None	Many
Jk	Anti-Jk^a	Few	All
MNSs	Anti-M, -N	None	None
	Anti-S,-s	None	Some
Lu	Anti-Lu^a, -Lu^b	None	None
Di	Anti-Dia	Few	Few
Xg	Anti-Xg^a	None	Many

- About 10–20% of patients with warm autoimmune hemolytic anaemia (AIHA) demonstrate *in vivo* sensitization with complement component C3.
- In cold hemagglutinin disease, the cold reactive autoantibody can react with red blood cell antigens at temperature up to 32°C range (thermal amplitude 10–32°C). When the patient is exposed to cold temperature, red blood cell is sensitized with autoantibody, which activates complement. If the red blood cells escape haemolysis, they return to the central circulation where the temperature is 37°C and the autoantibody dissociates from the cells, leaving complement component C3d firmly bound to the red blood cell membrane.

BASIC CONCEPTS OF ANTIHUMAN GLOBULIN TESTING

Two major classes of antibodies react with red blood cell antigens namely the complete antibodies and the incomplete antibodies.

Complete antibodies, or saline agglutinins will agglutinate red blood cells suspended in saline, which are usually IgM class of antibodies.

Incomplete antibodies do not react in saline but require special techniques to agglutinate red blood cells; which are IgG class of antibodies.

After combining with their corresponding antigen, blood group antibodies have the ability to activate complement. Thus, sensitization of incomplete antibodies or complement component can occur either *in vivo* or *in vitro*. Antihuman globulin reagent is used to detect IgG or complement sensitized red blood cells.

For detecting *in vivo* antibody or complement sensitization, direct antihuman globulin test (DAT) is used; whereas for detecting in vitro antibody or complement sensitization, indirect antihuman globulin test (IAT) is used.

DIRECT ANTIHUMAN GLOBULIN TEST

DAT is used to detect *in vivo* sensitization of red blood cells with immunoglobulin, complement or both. A positive DAT, with or without shortened red blood cell survival, may result from:

- Autoantibodies to intrinsic red blood cell antigens.
- Alloantibodies in recipients circulation reacting with antigens on recently transfused donor red blood cells.
- Alloantibodies in donor plasma, plasma derivatives or blood fractions, which react with antigens on red blood cells of transfusion recipients.
- Alloantibodies in maternal circulation, which cross placenta and sensitize fetal red blood cells (HDN).
- Antibodies directed against certain drugs, which bind to red blood cell membranes (e.g. Penicillin).
- Adsorbed proteins, including immunoglobulins, which attach to abnormal membranes or red blood cells modified by therapy with certain drugs, notably those of cephalosporin group.
- Complement components or rarely IgG bound to red blood cells after administration of drugs such as quinidine and phenacetin may induce drug antidrug interaction.
- Non-red blood cell immunoglobulins associated with red blood cells in patient with hypergammaglobulinemia or recipients with high dose of intravenous gamma globulin.
- In patient with organ transplantation, passenger lymphocytes of donor origin produce antibodies directed against ABO or other antigens on the recipient's cells, causing a positive DAT.
- Patients receiving antilymphocyte globulin (ALG) or antithymocyte globulin (ATG) of animal origin may develop a positive DAT within a few days, apparently related to high titer heterophile antibodies in these products and the presence of corresponding antibodies in animal-derived AHG sera.

MAJOR APPLICATIONS OF DAT IN BLOOD GROUP SEROLOGY

Hemolytic Disease of the Newborn (HDN)

In HDN fetal red blood cells *in vivo* are sensitized with IgG alloantibody of maternal origin thereby demonstrating a positive DAT with cord red blood cells. The most commonly observed HDN is due to Rho (D) incompatibility between mother and fetus.

If the father is Rho (D) positive and the mother is Rho (D) negative and during first pregnancy their progeny inherits Rho (D) positive red blood cell antigens, during parturition, the fetal red blood cells can enter mother's circulation providing antigenic stimulus for the production of anti-D antibodies. These anti-D antibodies normally will not have any

effect during the first Rh-incompatible pregnancy unless the mother has anti-D antibodies by previous incompatible blood transfusions.

During subsequent pregnancy, for the same couple, if the fetus is Rho (D) positive again, the anti-D antibodies will be activated along with the presence of anti-D antibodies from the first pregnancy already in the circulation. Since the IgG antibodies cross the placental barrier, these circulating anti-D will sensitize and destroy fetal Rho (D) positive cells. This process is demonstrated by a positive DAT on cord red blood cells (Fig. 25.13).

Transfusion Reactions

A patient will demonstrate positive DAT, if serum contains antibodies against red blood cell antigens of donor red blood cells. Likewise antibody present in donor plasma may also react with recipient red blood cells thereby demonstrating positive DAT.

Other Immune Hemolytic Diseases

A positive DAT may be observed due to acquired hemolytic anemia probably because of autoantibodies directed against individual's own intrinsic red blood cell antigens.

Classification of Autoimmune Hemolytic Anemia

- Warm autoimmune hemolytic anemia (WAIHA)
  - Primary (idiopathic)
  - Secondary (to conditions such as lymphoma, SLE, carcinoma, drug therapy)
- Cold agglutinin syndrome (CAS)
  - Primary (idiopathic)
  - Secondary (to conditions such as lymphoma, *Mycoplasma pneumoniae*, infectious mononucleosis)
  - Mixed type Autoimmune hemolytic anemia
  - Primary (idiopathic)
  - Secondary (to conditions such as SLE, lymphoma)
- Paroxysmal cold hemoglobinuria (PCH)
  - Primary (idiopathic)
  - Secondary (to conditions such as syphilis, viral infections)
- DAT negative autoimmune hemolytic anemia
  - Primary (idiopathic)
  - Secondary (to conditions such as lymphoma, SLE)

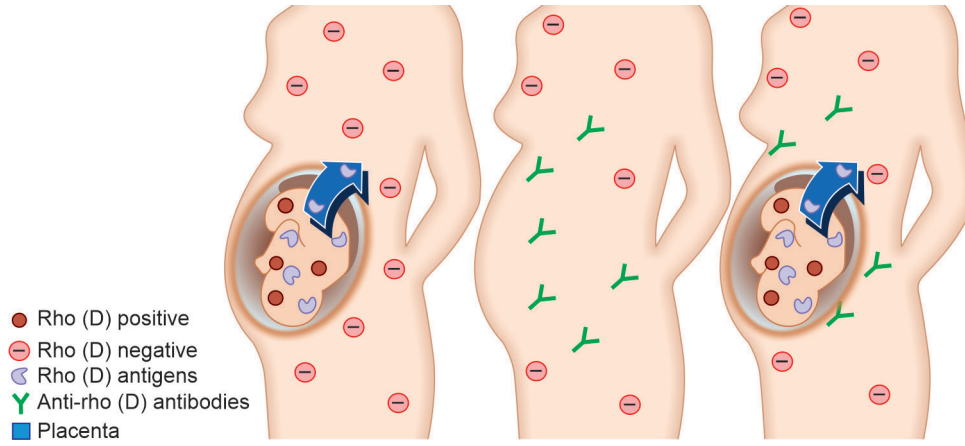


Fig. 25.13: Illustration of HDN

Drug-induced Hemolytic Anemia

Also, certain drugs namely penicillin, procainamide, cephalosporins may also be associated with immune red blood cell destruction thereby demonstrating a positive DAT (Fig. 25.14).

Importance of Serological Studies in DAT Positive Results (Table 25.6)

As per blood bankers' technical manual, three investigation approaches are helpful in evaluation of positive DAT:

- Test the DAT positive red blood cells with monospecific antihuman IgG and monospecific antihuman C3d reagent to characterize type of proteins sensitized with red blood cell membrane,
- Test serum/plasma to detect and identify clinically significant antibodies to red blood cell antigens.
- Test eluate prepared from sensitized red blood cells with a panel of reagent red blood cells to define whether the sensitized

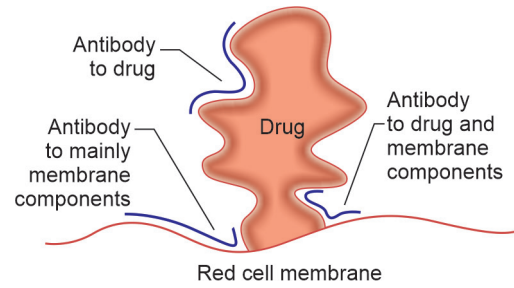


Fig. 25.14: Illustration of drug-induced antibody reactions

Table 25.6: Probable serological findings with DAT positive—AIHA / drug induced hemolytic anemia

Parameter	*WAIHA*	*CAS*	*Mixed type AIHA*	*PCH*	*Drug-induced AIHA*
DAT positive result	IgG/IgG + C3/C3	Mostly C3	IgG + C3	Mostly C3	IgG/IgG + C3
Immuno-globulin type	IgG (sometimes IgA or IgM rarely alone	IgM	IgG, IgM	IgG	IgG
Eluate	IgG	Nonreactive	IgG	Nonreactive	IgG
Serum	• May react by IAT • May hemolyze enzyme treated red cells at 37°C • Mostly agglutinate enzyme treated red cells at 37°C • May agglutinate untreated red cells at 20°C • Rarely agglutinate untreated cells at 37°C	• IgM hem-agglutinating antibody reactive at 4°C usually react at 30°C in albumin	• IgG IAT reactive antibody • IgM hem-agglutinating antibody usually react at 30–37°C in saline, also may react at 4°C saline	• IgG biphasic hemolysin (Donath–Landsteiner antibody)	• IgG antibody similar to WAIHA
Specificity	Usually Rh specificity	Usually Anti-I but can be Anti-i rarely Anti-Pr	Usually specificity unclear, can be anti-I, anti-i or other cold aggluthin specificities	Anti-P (non-reactive with p and Pk red cells	Specificity often Rh related

protein is immunoglobulin or complement component. Elution frees antibody from sensitized red blood cells and recovers antibody in usable form. When only complement is sensitized, eluates are frequently non-reactive.

INDIRECT ANTIHUMAN GLOBULIN TEST

In IAT procedures, serum or plasma is incubated with red blood cells, washed to remove unbound globulins. Agglutination that occurs after addition of antihuman globulin reagent indicates reaction between antibody in the serum and antigen present on the red blood cell membrane.

Applications of IAT

IAT determines *in vitro* sensitization of red blood cells and is used in the following situations:

- Detection of incomplete antibodies to potential donor red blood cells, pregnant women, blood donors.
- Identification of antibody specificity using a panel of red blood cells with known antigenic profile.
- Determination of red blood cell phenotype using known antisera (e.g. Du testing)
- Titration of incomplete antibodies.

Probable Sources of Error in Antihuman Globulin Testing

False-negative Results

- Neutralization of antihuman globulin reagent
- Failure to wash cells adequately to remove all serum/plasma. Fill tube at least three fourth full of saline for each wash.
  - If increased serum volumes are used, routine wash may be inadequate. Wash additional times more than three or four wash phases.
  - Contamination of antihuman globulin reagent by extraneous protein. Do not use finger or hand to cover tube. Contaminated droppers or wrong reagent dropper can neutralize entire vial of antihuman globulin reagent.
  - High concentration of IgG paraproteins in test serum (cryoglobulin). Wash additional times more than three or four wash phases.
- Interruption in testing
  - Bound IgG may dissociate from red blood cells or leave too little IgG to detect or may neutralize antihuman globulin reagent. Perform the test immediately.
  - Agglutination of IgG coated cells will weaken. Centrifuge and read immediately.
- Improper reagent storage
  - Antihuman globulin reagent may loose reactivity if frozen. Reagent may become bacterially contaminated. Store at the recommended storage condition.
  - Excess heat or repeated freeze/thaw cycles may cause loss of reactivity of antihuman globulin reagent. Replace the reagent back to the recommended storage condition.
- Improper procedure
  - Overcentrifugation may pack cells so tightly that agitation required to resuspend cells breaks up agglutinates.
  - Undercentrifugation may not be optimal for agglutination. The optimum centrifugation speed should be ascertained for each centrifuge.
  - Failure to add test serum, enhancement medium or antihuman globulin reagent may lead to negative test result. Follow the manufacturer's instructions meticulously.

- Too heavy red cell concentration may mask weak agglutination. Too light suspension may be difficult to read.
- Improper/insufficient serum-cell ratio.

- Complement
  - Rare antibodies, notably anti-Jka and anti-Jkb may only be detected when polyspecific antihuman globulin reagent is used and active complement is present.
- Saline
  - Low pH of saline solution can decrease sensitivity of antihuman globulin test.
  - Optimal wash solution for most antibodies is pH 7.0–7.2. It has been observed that commercially available infusion saline/saline stored in plastic containers can seriously compromise the sensitivity of antihuman globulin test. Saline stored in plastic containers and further autoclaved leads to leaching of certain chemicals which shifts the pH to the acidic side and impacts the sensitivity of antihuman globulin test. Preferably, use phosphate buffered saline as wash solution or suspending medium.
  - Some antibodies may require saline to be at specific temperature to retain antibody on red blood cell. Use 37°C or 4°C saline.

False-positive Results

- Particles or contaminants
  - Dust or dirt in glassware may cause clumping of cells. Fibrin or precipitates in test serum may similarly produce cell clumps that mimic agglutination.
- Improper procedure
  - Overcentrifugation may pack cells so tightly that they do not easily disperse and appear positive.
  - Centrifugation of test with polyethylene glycol or positively charged polymers prior to washing may create clumps that do not disperse.
- Cells with positive DAT result
  - Cells that are positive by DAT will also be positive in any indirect antiglobulin test. In such cases, antibodies should be eluted from the sensitized cells.
- Complement
  - Complement components, primarily C4, may bind to cells from clots or from CPDA-1 donor segments during storage at 4°C and occasionally at higher temperature. For DATs, use red blood cells anticoagulated with EDTA, ACD or CPD.
  - Samples collected in scratched glass tubes can lead to spurious activation of complement.
  - Complement may attach to cells in specimens collected from infusion lines used to administer dextrose-containing solutions. Strongest reactions are seen when large bore needles are used or when sample volume is less than 0.5 mL.

BLOOD TRANSFUSION

The procedures described earlier in this chapter are done mainly for providing appropriate blood for transfusion, i.e. the donor's blood should be compatible in all ways with the recipient's blood.

BLOOD DONORS

Most individuals can give blood without any ill effects, as a matter of fact, without any symptoms at all. The donors may be volunteers or paid donors. Blood transfusion should

always be a safe, harmless procedure, therefore, the donors must be screened. Two basic principles govern the transfusion services:

1. The donor should not be harmed in any way.
2. The recipient should be equally safe, i.e. the transfusion should be absolutely safe for the recipient too.

Donor Screening

1. *Identification:* Identify the donor name, address and other pertinent information. No donor should donate twice in three months' period. Preferably, the donors should be between the ages 18 and 60 years.
2. *History:* The questions should be guided about the present state of health; recent illness, if any; operations, if any; if the donor has ever been transfused; (for woman) if she ever gave birth to children who developed jaundice shortly after birth; if they have had malaria, jaundice, syphilis, tuberculosis; if they have heart disease, diabetes, etc. Donors may give wrong information on two accounts: (i) They want to donate blood for want of money. (ii) They do not want to donate blood for they are afraid of doing so. Hence, a battery of tests to be done becomes necessary.
3. *Physical examination:*
   - Temperature reading, (exclude those having fever).
   - Blood pressure should be normal or only slightly raised. Disqualify those who have moderate to marked hypertension.
   - Pulse should be normal without any irregularities.
   - Auscultate the chest for any respiratory or cardiac disorder. Discard individuals with respiratory/cardiac problem.
   - Pregnant women should not donate if they are anemic.
   - Finding of any venereal disease should outrightly disqualify the donor until he receives adequate treatment and gets rid of his problem.
4. *Hemoglobin:* The donors should not be anemic. It is best to disqualify anybody having Hb <12 g% but keeping in view our health, dietetic, and economic structure up to 10 g% can, however, be accepted, weighing the urgency of the demand for blood. As given in the hematology section specific gravity by copper sulfate method is adequate for screening donors' Hb.
5. *Malarial parasite:* Malarial blood can cause transfusion malaria if it is transfused. Screen all donors by making thick and thin smears of their blood. In some chronic malaria patient, no parasites may be seen on the peripheral smear examination but a unit of their blood would pass on enough parasites to the recipient.
6. *Microfilaria:* Screening for microfilaria should be done both by making a wet, coverslip mount of a drop of fresh blood (looking for the shipping movement of the microfilaria) and also on the direct and stained films used for searching malarial parasites. All microfilaria harboring donors are rejected.
7. *Icteric index:* This is the easiest method of measuring and quantitating the amount of pigment (mostly bilirubin) in the blood. Serum hepatitis is one of the most dreaded complications of transfusion. If a person has had jaundice during the last 5 years or is having it—he should be disqualified as a donor. History might be difficult to obtain and hence, liver function tests may be necessary where there is even slightest of doubt. The simplest and quickest to perform is icteric index, a measurement of the level of bilirubin, expressed in units based upon a comparison of the color of the serum and the color of a standard dilute solution of potassium dichromate. If the icterus index is >6 units, the donor should not be allowed to donate.

A major complication of transfusing blood from a hepatitis B/C patient or carrier is the transmission of the disease to the recipient.
Now ELISA, ICT techniques are available.

8. *Serology:* VDRL/Kahn's tests should be performed on blood of all donors. The blood positive for this test may pass on live spirochetes to the recipient. Weighing the need of blood even such a positive sample may be transfused and the recipient given a course of penicillin. In no case should any drug be put into the transfusion bottle. It is known that *Treponema pallidum* cannot survive for more than 48–72 hours with the condition of storage of anticoagulated blood in a refrigerator at 4°C. Within that time they are all dead. Hence, blood which has been stored as mentioned above cannot transmit syphilis since there would be no viable organisms remaining. A new syphilis patient's blood may be VDRL/Kahn's negative even though his blood would contain the spirochetes, hence, some believe in storing all blood for 48–72 hours before allowing it to be transfused. It is a good practice to indicate to the doctor who has requisitioned the blood, date and time of procuring that unit of blood.
9. Grouping and typing of donor: This has already been dealt with at great length earlier in this chapter.
10. Antibody screening: Ideally an indirect Coombs' test should be done on every donor to screen for atypical, incomplete antibodies.
11. HIV/AIDS screening is a must.
(Information regarding kits__refer to Serology chapter)

Drawing of Blood

1. Have the donor lie down.
2. Under aseptic conditions, do a venepuncture with a fairly large needle on the donor set-15 or 16 gauge needle, for gravity flow collection method. The bottle is kept below the level of arm and has an airway so that air may escape while the bottle gets filled with blood.
3. Where vacuum bottles are available a smaller gauge needle can be used. Do not puncture such a bottle.
4. Plastic bags containing the preservative fluid are already collapsed and hence, need no airway.
5. A unit of blood may mean different volumes at different centers. A standard unit is 420 mL of blood and 80 mL of ACD or CPD solution, i.e. a total 500 mL.
6. While the blood is being drawn, swirl the bottle gently to allow the ACD to act.
7. Flow of blood is usually hastened by: (i) having a blood pressure cuff on the arm above the needle, inflated to a pressure of 40–50 mm Hg and (ii) asking the patient to gently flex the fingers or clench and unclench his fist to assist the flow of the blood into the vein from which it is being taken.
8. When the full amount of donation has been collected, draw the needle from the bottle and put 5–10 mL samples into 2 or 3 sterile, dry tubes, each with the same number as that of the large blood transfusion bottle.
9. Now the needle from the vein and the blood pressure cuff can be removed.
10. Immediately apply pressure with cotton at the area, preferrably with the arm held straight up in an extended position. Have the donor hold it for minimum 3–5 minutes.
11. Close the bottle immediately after sterilizing the rubber cover.
12. Once closed, do not open and enter the bottle until the requisitioning doctor inserts the giving set. Be sure the bottle is completely and correctly labeled with Number, Name, Group, Type, Date, etc. Double check this.

13. Do not let the donor get up immediately. Let the donor's body adjust to the loss of this blood. Offering a nourishing drink and snacks to the donor is indeed a very good way of saying thanks to the donor. Encourage him to drink little extra water for a few hours after the donation.
14. The preservatives have been mentioned in the foregoing pages.
15. Store the donated blood at 4–6°C (37–42°F) in a refrigerator that should solely be used for keeping the blood transfusion bottles only. If this blood is kept constantly at the required temperature—a duration of 21 days may be allowed to lapse between collection and transfusion of the blood. If storage conditions are less ideal, then it is best to shorten the storage time to about 14 days (after this duration it should not be transfused).

Adverse Donor Reactions

Problems with Blood Flow

Occasionally, venepuncture is unsuccessful or the vein may develop spasm after venepuncture so that blood flow is not maintained.

If this happens:

1. Do not try to probe around in the vein, as this can result in a hematoma and discomfort for the donor.
2. Remove the needle and discard the pack as it will be contaminated.
3. Never re-site the needle in the same arm.
4. Reassure the donor, giving a full explanation for the unsuccessful venepuncture in order to retain their confidence, and apologize.

If the donor consents, a further venepuncture on the other arm may be attempted, provided that a suitable vein is located. No more than a total of 350/450 mL of blood should be with-drawn from both sides.

If there is a failure to maintain a blood flow during the collection, the person who has performed the venepuncture should be informed immediately. Slowing of the flow rate may be due to:

- Reduced cuff pressure: Check that cuff pressure has been maintained
- Occlusion of the lumen of the needle by the vein wall: Rotating the needle may help
- Positioning of the lumen of the needle on a valve within the vein: Try to reestablish the flow by withdrawing the needle gently or even by slight rotation of the needle

Before doing any of these things:

1. Explain that there is a problem with the blood flow and ask whether the donor is experiencing any discomfort.
2. Remove the swab and check that there is no hematoma present.
3. If there are no other apparent problems, proceed with adjusting the needle.
4. Avoid excessive manipulation of the needle or squeezing the donor tubing as small clots may form which will then be released into the circulation.

A failure to reestablish a blood flow will result in a partial collection. This should be marked on the donor's record form and the donor should be given an explanation and apology, if the collection is too slow, the donation should be discontinued. This should be recorded on the donor's record form.

Hematoma

Hematoma can be prevented by good venepuncture technique and application of adequate pressure following donation.

If a hematoma is noted:
1. Stop the donation.
2. Apply firm pressure until the venepuncture site stops oozing blood.
3. Apply an anti-inflammatory cream in a circular motion over the area and cover it with a small plaster or swab dressing, then apply a pressure bandage.
4. Reassure the donor, explaining what has happened and the reason for the bruise, and then apologize.
5. Ask the donor to keep the plaster on for 24 hours and the bandage on for 2–4 hours. If they feel that it is too tight and stopping their circulation, it should be loosened.
6. Tell the donor that they can use their arm normally, but should not lift any heavy objects. Also, tell them that they can take painkillers for moderate discomfort, but that if the area becomes unduly painful, they should contact the transfusion center or their own doctor.
7. Record details of the hematoma on the donor's record form.

Accidental Puncture of the Artery

This is an uncommon complication of blood donation, and one should be able to recognize it immediately by a very fast flow of bright red blood.
If accidental puncture of the artery occurs:
1. Discontinue the donation immediately and apply hard pressure to the puncture site immediately after the withdrawal of the needle. Raise the limb above heart level.
2. Maintain pressure for a minimum of 15 minutes.
3. When the bleeding has stopped, apply a pressure bandage and tell the donor to keep this on for 4–6 hours.
4. Reassure the donor, giving a full explanation of what has happened, and apologize.
5. Record the appropriate information on the donor's record form.
6 Do not allow the donor to leave until they are feeling well and after the most senior member of the donor clinic staff has discharged them.
7. If you suspect that tissue bleeding may still be continuing, refer the donor to the nearest hospital or health center. If the donor lives near the donor clinic, ask him to come back for assessment the following day.

Mild, Moderate, or Severe Reactions

Most people can tolerate the withdrawal of 350 or 450 mL of blood without any ill-effects. Others experience reactions ranging in severity from a feeling of uneasiness' to obvious shock-like symptoms, fainting or even generalized convulsions. These reactions can occur at any time—during the donor selection process, during donation, in the resting or refreshment area or even hours following a donation.

There is a psychological element to most reactions, so a friendly, cheerful atmosphere at the session can often reduce donor anxiety and perhaps prevent any adverse reactions. Donor reactions do sometimes occur, however, and can be categorized as follows:
Mild: Vasovagal symptoms without loss of consciousness.
Moderate : A progression of symptoms associated with a mild donor reaction resulting in unconsciousness.
Severe: Any of the above, accompanied by convulsions (uncommon).

Mild Donor Reactions

The signs of mild donor reactions include:
- Anxiety
- Increased respiration

- Rapid pulse
- Pallor and mild sweating
  - Dizziness/continuous yawning
  - Nausea/vomiting

When Mild Donor Reactions Occur

1. Discontinue the donation.
2. Raise both of the donor's legs and lower the head to improve the blood supply. If a donor is vomiting, turn him on one side to avoid accidental inhaling of vomitus.
3. Loosen or remove tight clothing.
4. Keep the donor cool by opening windows or switching on a fan.
5. Have a suitable receptacle available at the bedside in case the donor vomits.
6. Allow a sufficient rest period.
7. Offer a cold drink.
8. Once the donor has recovered, assist him from the bed to the refreshment area where another cold drink should be given.
9. Reassure and talk to the donor throughout all these stages. Explain that this type of reaction is common and does not mean that they are now physically 'unwell'.
10. Record the reaction on the donor's record form.
11 Advise the donor that, if symptoms persist, they should report to the blood bank or consult a doctor.
12. Ensure that the donor is fully recovered before leaving the session and has been seen by a trained member of staff.

Moderate Donor Reactions

The signs of moderate donor reactions include:

- Loss of consciousness (fainting)
- Repeated periods of unconsciousness
- A slow pulse which may be difficult to feel because of poor volume
- Shallow respirations.

When Moderate Donor Reactions Occur

1. Discontinue the donation.
2. Raise both of the donor's legs and lower the head.
3. Ensure that the donor is examined by a medical officer or a senior nurse.
4. Loosen or remove tight clothing.
5. Keep the donor cool by opening windows or switching on a fan.
6. Have a suitable receptacle available at the bedside in case the donor vomits.
7. Check the pulse rate regularly. The appearance of the donor and the pulse rate are a good guide to the donor's condition.
8. If possible, remove the donor to another room for privacy and to prevent other donors from seeing what is happening.
9. If there is no other room available, put screens around the donor.
10. Ensure that someone remains with the donor.
11. Reassure and talk to the donor throughout all these stages. It may be necessary to advise the donor not to donate in future.
12. Record the reaction on the donor's record form.
13. Ensure that the donor rests for some time and is fully recovered before leaving.

14. Advise the donor that if symptoms persist, they should contact their doctor of the nearest hospital.
15. Ensure that the donor is discharged by a senior member of staff.
16. Where feasible, arrange transport home for the donor.

Severe Donor Reactions

A faint may be accompanied by convulsions. Convulsions may be preceded by all the signs and symptoms of a vasovagal attack or they may occur without warning. Convulsions vary in severity from loss of consciousness accompanied by a slight twitching of extremities to a grandmal: type seizure with incontinence of urine or feces. A medical officer or trained nurse must be called immediately.

Faints are common but convulsions are very uncommon. If the correct procedure for donor screening has been carried out through the medical history and health check convulsions should not occur. Most convulsions stop within a few minutes, but they are often very upsetting for other donors so staff not actively involved in looking after a convulsing donor should distract and reassure other donors.

When Generalized Convulsions Occur

1. Turn the donor to a lateral position to maintain a clear airway.
2. Gently restrain the donor to prevent from injury.
3. Put screens around the donor to maintain privacy.
4. Check the pulse rate frequently.
5. Ensure that the donor is examined by a medical officer.
6. Loosen tight clothing.
7. Keep the donor cool by opening windows or switching on a fan.
8. If a convulsion lasts longer than five minutes, this is a medical emergency and a medical officer must be in attendance. Valium may be given intravenously under the direction of the medical officer. IM valium is ineffective in these circumstances.
9. Reassure the donor and explain what has happened.
10. Tactfully advise the donor not to donate blood again.
11. Record the incident:
    - On the donor's record form.
    - In the blood bank incident book.
12. Recheck the donor's medical history and record of the predonation health check to identify whether there were any indications that a convulsion might occur.
13. Advise the donor that they should contact their doctor or the nearest hospital.
14. Ensure that the donor rests for some time and is fully recovered before leaving the session.
15. Ensure that the donor is discharged by a medical officer or a very senior member of staff.
16. Inform the donor's own doctor about the incident.
17. Arrange transport home for the donor when fully recovered and ensure that they are escorted or arrange for their transfer to hospital.

Hyperventilation

Hyperventilation is a rapid overbreathing which lowers the carbon dioxide content of blood. In turn, this leads to muscle spasms. Talking to the donor to reassure him and relieve anxiety should prevent hyperventilation.

If hyperventilation occurs:

1. Instruct the donor to breathe quietly and slowly, but not deeply.
2. If this fails to relieve muscle spasms, instruct the donor to rebreathe expired air into a paper bag.
3. Explain what is happening and reassure the donor.

Accidents

There may be a risk of injury to the head or body if a donor faints and falls

When head injuries or other injuries requiring medical attention occur:

1. Always ensure that the donor is examined by a medical officer or senior nurse.
2. If there is any doubt about the donor's condition, arrange for his transfer to hospital with a doctor or qualified nurse as escort.
3. *Record the incident:*
   - On the donor's record form.
   - On an accident form (this should also be filled in if a member of staff is involved in an accident)
   - In the blood bank incident book.

If the Injury is of a minor nature:

1. Ensure that the donor rests for some time and is fully recovered before leaving, unless transfer to hospital has been arranged.
2. Ensure that the donor is discharged by a senior member of staff who should decide whether the donor's own doctor should be informed.
3. Advise the donor that if he feels unwell, he should contact his doctor or the nearest hospital.

COMPATIBILITY TESTING

Cross-match

The final green signal for allowing blood transfusion is compatibility between the donor's and recipient's blood. This is done *in vitro* in the laboratory/blood bank. A compatibility test is mandatory before all transfusions.

Cross-matching is done in two parts: (i) The major cross-match and (ii) The minor cross-match. In the former the donor's cells are mixed with the patient's serum and in latter the patient's cells are mixed with the donor's serum (Fig. 25.15).

Saline Cross-match

Saline suspension of the cells is mixed with serum. This is done at room temperature and only complete, saline active, 'cold' antibodies will be detected.

Open-slide Method

1. Label one tube with patient's name or number (number is preferable as there may be many patients with the same name).
2. Label another tube with the donor's name or number one for each bottle of blood to be crossmatched.
3. Half-fill each tube with saline.

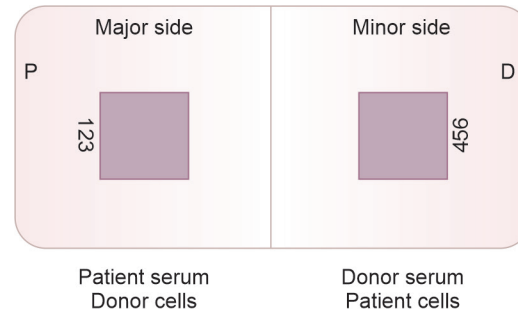


Fig. 25.15: Cross-matching

4. Label one slide for each cross-match. Draw a line in the middle to separate the two sides.

   Put the patient's number on the left side and mark it 'P' on that side. This is the major side which uses the patient's (P) serum. Put the donor's number on the right side and identify it further by marking it 'D'. This is the minor side, which uses the donor's (D) serum. Draw two circles one on each side with a glass marking pencil (Fig. 25.15).
5. Make 5% red cell suspension in each of the labeled tubes—patient's and the donor's bloods. (Get the donor cells and serum from one of the 'pilot' tubes filled at the end of the donation of blood). Do not enter the bottle of ACD blood for this purpose unless absolu- tely necessary. If you do enter the bottle, you have almost inevitably introduced bacteria— even if you have been careful—and that blood should be used immediately or not at all.
6. To the patient's (P), major side of the slide, add one drop of patient's serum and one drop of the donor's cell suspension
7. To the donor's (D), minor side of the slide, add one drop of donor's serum and one drop of the patient's cell suspension.
8. Mix by gently rotating the slide and incubate at room temperature for 10–15 minutes. (Drying will make the results difficult to interpret. Drying can be retarded if the slides are kept in a petridish).
9. Examine both macroscopically and microscopically for agglutination (by gently rotating and tipping the slide).

This is a simple and a rapid method. However, incompatibilities due to weak saline agglutinins and all incomplete immune antibodies will not be detected. The period of incubation should not be reduced or else agglutination may not occur. Almost all ABO group incompatibili- ties will be detected by this method. Rh incompatibilities will never be detected.

Saline Tube Method

1. Label one tube for the patient's 2–5% saline cell suspension. Label one tube for each donor's saline cell suspension. Label two tubes for each cross-match—one marked P with patient's number and donor's number, and one marked D with donor's number and patient's number.
2. Add saline to the cell suspension tubes and make the cell suspensions.
3. To the patient's tubes (P. major side), add one drop of patient's serum and one drop of donor cell suspension.
4. To the donor's tubes (D. minor side) add one drop of donor's serum and one drop of patient cell suspension.
5. Mix the cells and sera by gently tapping the tubes.
6. Let stand at room temperature for 30 minutes.
7. Examine for agglutination both macroscopically and microscopically.

This method needs a longer incubation period but has the advantage of detecting weak reactions more often. However, it still will only detect complete agglutinins—not immune antibodies of the Rh type.

Immediate-spin and Thermal Incubation Modification

1–5. As in the previous method.
6. After 2–3 minutes at room temperature, centrifuge at 500–1000 rpm for 2 minutes.

7. Remove and examine macroscopically, holding the bottom of the tube over a concave mirror (a microscope mirror is good enough) so that you can observe the button of cells at the bottom when you tap the tube gently to resuspend the cells. If the cells show agglutination, check them microscopically by placing a drop on a slide. This indicates incompatibility and the cross-match result can be recorded. If the cells resuspend without any clumps, then proceed to the next step.
8. Incubate the tube for 60 minutes at 37°C.
9. Examine the cells for agglutination, both, macroscopically and microscopically. Centrifugation brings the cells into close proximity and hastens agglutination where it is going to occur—the immediate spin. Incubation at 37°C may detect some complete warm antibodies active in saline but missed in room temperature incubations.

Albumin Tube Method

1. Set up the tubes as for saline method.
2. Incubate the cells and serum for 60 minutes at 37°C—thermal saline incubation.
3. Allow a drop of albumin to run down the inside of each cross-matching tube so that it forms a layer between the cells and serum (human/bovine albumin, 22–30%).
4. Incubate at 37°C for an additional 30 minutes.
5. Dislodge the cells gently and look for agglutination microscopically. This method will pick up some incompatibilities not detected in saline due to incomplete, immune antibodies, such as Rh antibodies.

Coombs' Cross-matching

1. Wash the patient's and donor's cells three times in saline. Then make 5–10% suspension of each in saline.
2. To labeled patient's tubes (P. major side) add 2 drops of patient's serum and 2–4 drops of donor cell suspension.
3. To labeled donor's tube (D. minor side) add 2 drops of donor's serum and 2–3 drops of patient cell suspension.
4. Incubate tubes at 37°C for one hour.
5. Centrifuge, decant the serum and wash the cells thrice in saline.
6. Add Coombs' serum to each tube and after letting stand for 5 minutes at room temperature, centrifuge at 500–1000 rpm for 2 minutes.
7. Examine the cells macroscopically and microscopically.

The Coombs' serum in the cross-match as in the indirect Combs' test, will detect the presence of incomplete, immune antibodies such as Rh-antibodies and show any Rh incompatibility. Remember that not all Rh negative persons have anti-Rh (D) antibodies—in fact only very few do—so that not all cross-matches of Rh-negative recipients with Rh-positive donors__or *vice versa*—will be incompatible. Transfusion of the blood may be perfectly safe. However, the Rh-positive transfused cells may sensitize the recipient so that he or she develops anti-Rh antibodies. On subsequent cross-matches (after 2–3 weeks), an incompatibility would be demonstrated. Thus, where Rh-negative individuals have been previously transfused, it is important to do a more sensitive cross-match, since there is a good chance that the previous transfusion may have been with Rh-positive blood.

It is obvious that cross-matching of bloods of the same groups generally gives a compatible result (major and minor). Cross-matching of O group blood with any other group gives a compatible major side (hence, O group people are called universal donors) but always gives an incompatible minor side. Any group of blood may be given to AB

group of patient with a compatible major cross-match (universal recipients) but again there is an incompatible minor cross-match.

Diagrammed results of the cross-matching of the different groups of blood (+ = agglutination, – = no agglutination) are described in Fig. 25.16.

Blood which shows a major incompatiblity should never be transfused (in the patient's body the 'major side' would be the full plasma volume—about 2,500 mL of antibodies and the transfused donor cells—200–250 mL). The minor cross-match is important but not as important as the major side (in the patient's body, the minor side would be the donor plasma volume 250–300 mL of antibodies and the large number of his own cells—about 2500 mL. The donor plasma becomes diluted in the much larger volume of the patient's plasma, and thus, the donor antibodies become diluted and dispersed so that although a few cells may be coated or agglutinate, there is no major reaction). In an emergency, therefore, it is possible to use blood which is minor incompatible (but major compatible) without leading to transfusion reaction. Sometimes, however, one can get into difficulty by transfusing blood which shows a minor incompatibility as given here.

High titer of donor antibodies: If the titer (amount) of antibodies in the donor blood is very high, then even if there is dilution of the donor's plasma in the patient's plasma, the antibodies may still be present in significant numbers to cause agglutination, hemolysis and result in a reaction. O donors with high titer are said to be dangerous universal donors.

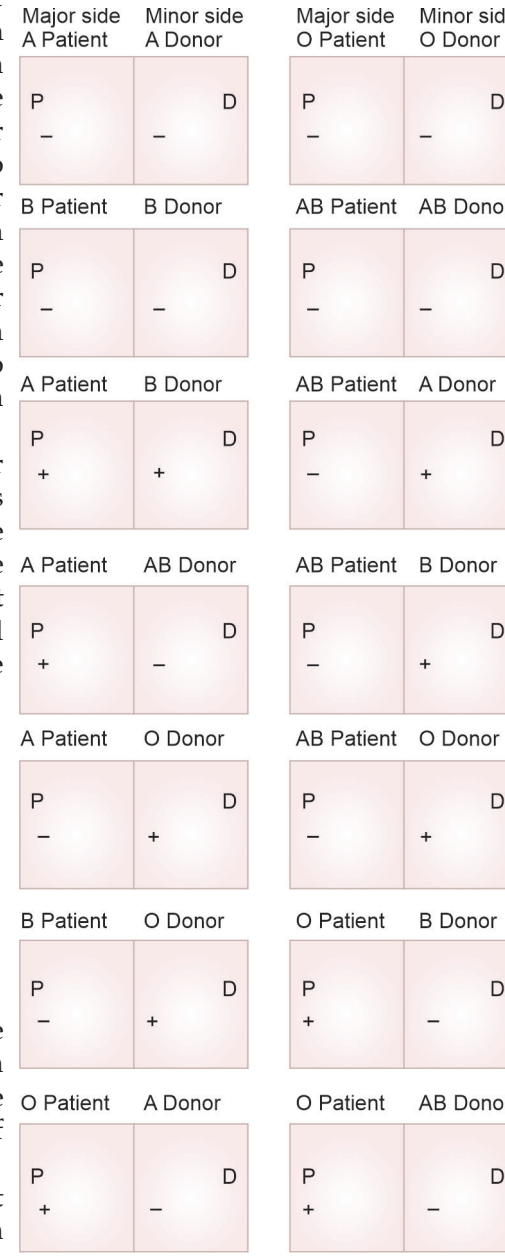


Fig. 25.16: Method of cross-matching universal donor blood

Method of Cross-matching Universal Donor Blood

1. Do major cross-match as described (Fig. 25.16).
2. Make a 1:100 dilution of donor serum in saline.
3. Use this diluted serum for a minor cross-match.
4. If there is no agglutination on minor side using the 1:100 diluted serum, then this a low titer (<1:100) plasma and can safely be transfused as long as too many bottles of such blood are not needed.
5. If there is agglutination on the minor side, it means that this is high titer (>1:100) plasma and should better not be used especially if more than one unit is required (for AB

patient, it is possible to use A, B, or O donor blood. Whichever is given, it should be crossmatched in this way).

Factors Leading to False Results

1. Autoagglutination.
2. Cold antibodies.
3. Bacterial contamination.
4. Drying.

If Still an Unexpected Incompatibility is Obtained

1. Test for autoagglutination.
2. Test for cold antibodies.
3. Regroup the patient and donor cells.
4. Back-type (serum type) the patient and donor serum.
5. Repeat the cross-match by tube technique.
6. Check the records to see if patient's present grouping is correct.
7. Ask for a fresh patient specimen if necessary.

CHOICE OF MATERIAL FOR TRANSFUSION

1. *Shock due to hemorrhage or decreased blood volume:* Whole blood fresh, stored or preserved.
2. *Shock resulting from trauma, burns, or infection:* Plasma liquid, frozen or dried; plasma substitutes.
3. *Chronic anemia:* Whole blood or resuspended red blood cells.
4. *Leukopenia:* Large amounts of fresh whole blood. Leukocyte concentrates.
5. *Prothrombin factor deficiency not corrected by vitamin K:* Fresh whole blood.
6. *Hemophilia:* Factor VIII (AHF) cryoprecipitate concentrate. Factor IX complex concentrate. Whole blood or plasma administered within 6 hours of collection.
7. *Thrombocytopenia:* Platelet transfusion with fresh (<3 days old) platelets.

BLOOD AND ITS PRODUCTS

Whole Blood

In most instances whole blood is requisitioned by the concerned physician/surgeon, except in certain circumstances when a special preparation may be asked for.

Fresh Blood

Stored bank blood contains nearly all of the substances required in a usual transfusion therapy. A few labile substances are lost.

1. Platelets: These stick to the rubber and glass surfaces and also die soon or become non- functional. If platelets are needed, fresh blood is necessary.
2. *WBCs:* These die fairly rapidly. On occasions where the patient is suffering from severe leukopenia with infection, a transfusion of fresh blood provides viable leukocytes which help to fight the infection but which do not raise the WBC count.
3. *Factor VIII:* Antihemophilic globulin. Patients with hemophilia should be transfused with fresh blood if they need transfusion to help stop bleeding, since AHG disappears rapidly from stored blood.
4. *Other labile factors:* Can be provided by giving frozen or dried plasma.

Packed Cells

Sedimented cells: The bottle kept in the refrigerator shows at bottom settled cells and above it the plasma. In chronic anemia one may want to transfuse just the packed cells. This

can be prepared by aspirating the plasma into a separate sterile bottle, leaving the cells in original bottle. Lable the plasma bottle with the date, the bottle number and the group and type of the blood from which it is obtained, aspirate a small amount of plasma into a 'pilot' tube for later cross-matches.

The packed cells should be transfused within 4 hours. Aspirate plasma only when someone comes to collect it, this ensures that the packed cells would be used immediately.

Plasma

The ACD plasma aspirated from the sedimented cells may be stored for months at 4–6°C and is safe for transfusion. If the plasma becomes cloudy or shows floating granules (bacterial colonies) suck little bit of it from the top, do a hanging drop and Gram's stain of the same—discard if it is found contaminated. Minor cross-matching should be done with the patient's red cells and the donor's plasma.

Plasma may be used as:

1. *Liquid plasma:* It should contain 5% dextrose to prevent precipitation of fibrin at room temperature. The prothrombin titer diminishes rapidly after 72 hours. Liquid plasma may be kept at room temperature for 3 years. Hepatitis virus is attenuated or destroyed in plasma that has remained at room temperature for 6 months.
2. *Frozen plasma:* Plasma frozen within 72 hours after blood is drawn may be stored indefinitely at –20°C (–4°F). Reliquefy at 37°C (98.6°F) in a water bath and use promptly. Frozen plasma retains its full content of labile constituents (prothrombin, complement, antibodies). Hepatitis virus is also preserved, frozen plasma should therefore not be used unless no other substitute for blood is available.
3. *Dried plasma:* Plasma dried after freezing within 72 hours after blood is drawn is stable at room temperature for 5 years if kept in an airtight container. Reliquefy with 0.1% solution of citric acid and administer within 1 hour. Dried plasma retains its full content of labile constituents. Hepatitis virus is preserved too.

BLOOD TRANSFUSION COMPLICATIONS

Complications Appearing Early

1. Hemolytic reaction:
   - Immediate or
   - Delayed.
2. Reactions due to infected blood.
3. Allergic reactions to white cells, platelets or proteins.
4. Pyrogenic reactions (febrile reactions)
   - Pyrogens may come from the bottle, tubing, or the blood itself. Precaution should be taken that equipment, solutions, etc. used should be absolutely sterile.
   - Bacterial contamination too would lead to pyrogenic reaction.
5. Circulatory overload
6. Air embolism
7. Thrombophlebitis
8. Hyperkalemia
9. Citrate toxicity
10. Clotting abnormalities (after massive transfusion).

Complications Appearing Late

1. Disease transmission, e.g.
   - Hepatitis B/C
   - Syphilis
   - Malaria
   - Cytomegalovirus
   - AIDS
2. Transfusional iron-overload
3. Immune sensitisation.

INVESTIGATIONS IN A CASE OF TRANSFUSION REACTION

The occurrence of a transfusion reaction should be immediately reported to the blood supplying laboratory or bank.

The reporting authority should send

1. A post-transfusion blood sample
2. A post-transfusion urine sample
3. A pretransfusion blood sample
4. If the blood has been discontinued, the bottle and the tubing intact should also be sent.

The laboratory or the bank providing the blood should already have

1. The patient's original cross-match specimen (these should be preserved for at least 48 hours after dispatching the blood or its products).
2. The donor's pilot tube/bottle (also to be preserved for 48 hours as mentioned above)
3. All the laboratory/bank records.

Proceed as Mentioned Below

1. Inspect the post-transfusion urine sample for the presence of hemolysis—hemoglobinuria. Centrifuge the specimen to see if the red color stays in the supernatant (hemoglobinuria) or goes down with the sediment (hematuria).
2. Centrifuge the post-transfusion blood specimen. Inspect the serum for the presence of hemolysis. If hemolysis has been established report it to the concerned physician/surgeon so that he may treat the patient as a case of hemolytic transfusion reaction.

To Establish the Cause of Hemolysis:

3. Regroup and retype the original donor pilot blood sample, the original patient's cross-match sample, the blood in the blood bottle or tubing.
4. Group and type the new patient's pretransfusion blood sample (if available—and post-transfusion specimen).
5. Recross-match—if available use combined saline and Coombs' cross-matches—the donor blood with the original patient specimen.
6. Cross-match the donor blood with the new patient specimen.
7. Make hanging drop preparation and Gram's stain of blood in tubing or blood bottle looking for bacteria. Culture the blood in the bottle or tubing.
8. Schumm's test—a spectroscopic examination of plasma for the bands which are typical of methemalbumin—found when there has been intravascular hemolysis.

Interpretation of Results

1. If there was no evidence of hemolysis in the blood and no free hemoglobin in the urine, the patient has not had a hemolytic reaction, but a pyrogenic/allergic reaction.
2. If there is evidence of hemolysis in the blood and hemolysis in the urine (hemoglobinuria), a hemolytic transfusion reaction has occurred.

3. If the recross-matching shows incompatibility, then the first cross-match was done or recorded in error.
4. If the recross-match with the original patient specimen is compatible, but the cross-match with the new patient specimen is incompatible, the mistake lies in mistaken identification of the patient, either when the first sample for cross-match was done, or at the time of giving the transfusion.
5. If the saline cross-matches are compatible but the Coombs' cross-matches are incompatible then the problem lies with an immune antibody incompatibility—the most common being the Rh incompatibility.

LABORATORY DIAGNOSIS OF HEMOLYTIC DISEASE OF THE NEWBORN

Laboratory findings at birth:

1. *Cord blood:*
   - Variable anemia (Hb <18 g%)
   - Reticulocytosis
   - Hyperbilirubinemia
   - Positive direct Coombs' test
   - Baby is Rh positive.
2. *The mother:*
   - is Rh negative, and
   - has a high plasma titer of anti-D.

TROUBLESHOOTING

GENERAL INSTRUCTIONS FOR BLOOD GROUPING

Sample Preparation

- Depending upon whether serum or plasma is to be used as sample for testing, blood may be collected with or without an anticoagulant. Serum vs. Plasma:
  For blood grouping tests, serum is preferred to plasma for the following reasons:
  1. Plasma samples may clot when incubated at 37°C
  2. The detection of some antibodies depends upon complement activation and anti-coagulants such as citrate of EDTA prevent complement activation by chelating calcium.
- Containers for blood collection and processing should be clean and dry, free of detergents; acids and alkalies, ideally made of plastic or siliconized glass tubes.

Sample Processing

- Need to wash red cells (tube test)
- Red cell suspension used in blood grouping should be washed free of their own plasma. If this is not done clots will form when the red cell suspension, which contains fibrinogen, is mixed with serum, which contains residual thrombin.

Other reasons for washing red cells are as follows:

1. Plasma tends to cause rouleaux formation, which interferes with the interpretation of agglutination tests.
   Rouleaux or pseudoagglutination is a phenomenon characterized by a person's serum causing his own and other red cells to line up in formations which resemble stacks of coins. This is easily mistaken for true agglutination.

Causes of Rouleaux:

a. Concentration of serum.
b. Increase of plasma proteins.
c. The transfusion of macromolecular medium, e.g. dextran.
d. Inverted albumin/globulin ratio as in chronic nephritis, kala-azar, and multiple myeloma.
e. Infections with an increased red cell sedimentation rate.

2. Plasma contains anticoagulants, which are anticomplementary and may thus interfere with the detection of complement binding antibodies.
3. Preservative substances added to red cell suspension, e.g. lactose or neomycin, are occasionally responsible for agglutination due to the presence of a corresponding antibody in the patient's plasma; most of the antibodies concerned do not react with red cells washed in saline.
4. Plasma contains blood group substances corresponding to those on the red cells and these substances may inhibit the antibody in the test serum.
5. Plasma may contain so-called albumin autoagglutinins and may then cause false-positive reactions when whole blood is added to a serum-albumin mixture.

Sample Storage

- Effect of storage on red cell antigens:
  1. Red cells stored as clotted blood lose their antigenic activity more rapidly than when stored with citrate anticoagulant.
  2. Similarly, when blood is collected into plastic bags, if the donor line is not emptied immediately after collection and then refilled with blood mixed with anticoagulant, the clotted blood in tubing is an unreliable source of red cells for cross-matching tests.
  3. Red cells stored as clotted blood may give false-positive reactions in the antiglobulin test due to uptake of complement components during storage at 4°C.
  4. Reagent red cells may be stored as whole blood with CPD or ACD but more usually they are stored as washed cells in a preservative solution. A modified Alsever's solution, with added inosine and with antibiotics is commonly used permitting satisfactory storage for at least 35 days at 4°C.
- Clotted whole blood should be tested within 14 days
- Anticoagulated blood using various anticoagulants should be tested within specific time limits as follows:
  - EDTA or heparin: 2 days
  - Sodium citrate or sodium oxalate: 14 days
  - ACD or CPD: 28 days

Equipments

- Prior to testing, check whether pipettes and other instruments used are well calibrated and in proper working condition.

Reagents

- The blood grouping reagents contain 0.1% sodium azide as preservative. Avoid contact of the reagent with skin and mucosa. On disposal flush with large quantities of water.
- Adhere to appropriate storage conditions of the reagent as mentioned in the respective package inserts.

Common Causes of False-negative and False-positive Results in ABO Testing

False-negative Results

- Reagent or test serum not added to a tube
- Hemolysis not identified as a positive reaction
- Inappropriate ratio of serum (or reagent) to red cells
- Tests not centrifuged sufficiently
- Tests incubated at temperatures above 20–24°C
- Incorrect interpretation of test results.

False-positive Results

- Overcentrifugation of tubes
- Use of contaminated reagents, red cells or saline
- Use of dirty glassware
- Incorrect interpretation of results

Interpretation of Agglutination Reactions

To avoid false readings and to standardize recording of results, tests should be read with the aid of the microscope. See table below:

++++	One complete mass of agglutination, readily visible on the slide before microscopic examination.
+++	Large separate masses of agglutination, readily visible on the slide before microscopic examination.
++	Smaller agglutinates, still readily visible on the slide before examination under the microscope.
+	A granular appearance, just visible on the slide before examination under the microscope. The microscope reveals big clumps of agglutinates of more than 20 cells.
(+)	Smaller clumps (12–20) cells only detectable by use of microscope.
GW	Good weak—clumps of 8–12 cells only detectable microscopically.
W	Weak—Weak reactions with uniform distribution of small clumps of 4–6 cells.
?	Sticky—uneven distribution of cells, 2 or 3 sticking together, more noticeable when the cells are "rolling" on the slide.
0 or –	All cells free and evenly distributed. The 0 sign is preferred since—can easily be altered to +.
MF/NF	Mixed field or negative field—agglutinates in a field of free cells, e.g. the type of reaction observed with mixed bloods

ABO GROUPING

Problem: False-positive Results

Possible causes	*Solutions*
1. Bacterial contamination (some bacteria such as *Staphylococcus aureus* will agglutinate all red cell samples thereby giving false-positive results)	Check the reagents for turbidity. Extreme turbidity may indicate bacterial contamination. Such contaminated reagents should not be used for testing

Contd.

Possible causes	*Solutions*
2. The antisera may be contaminated with another antibody. There may have been accidental mixing of antisera; this could be due to the interchanging of cap, e.g. if either anti A, anti B or anti A, B is contaminated with anti D as anti-D reagent is color-less and contamination is not visually observed in such a case the anti A or anti B or anti A, B reagent will give false positive results with all Rh +ve blood groups.	Check the color of the cap on the antisera bottle. Also check the antisera with the blood of a person whose blood group is known. If anti A, anti B or anti A, B vial is contaminated with anti D, then the anti A, anti B or anti A, B should be checked with Rh –ve blood sample, if it gives no agglutination thereby confirming anti D contamination or else it could be some other contamination
3. Particles of dust, debris, chemicals or detergents on the slide or in the tube giving nonspecific agglutination	Clean and dry glassware should be used while carrying out the test
4. Peripheral drying or fibrin strands were mistaken for agglutination in case of slide test	The test should not be carried out directly under the fan. The vials should be capped immediately after use. The results of the test should be read at 2 minutes not beyond as drying may be interpreted as test positive result. Except in case of anti A_1 lectin, the agglutination should be observed at one minute
5. Excessive centrifugation in case of tube test	Ensure that centrifugation is carried out for either one-minute at 1000 rpm or 20 seconds at 3400 rpm. Centrifugation should be adequate to produce a cell button with a clear supernatant but without packing the cells so tightly that they are difficult to dislodge.

Problem: False-negative Results

Possible causes	*Solutions*
1. Storage of antisera at higher/lower temperatures than specified	Reagents should be stored at 2–8°C when not in use. Thermal damage due to faulty storage may result in a loss of reactivity. Check the turbidity of the reagents; extreme turbidity may also be due to thermal damage. Antibody activity decreases at lower temperatures. Do not freeze the reagents
2. Blood sample stored for too long	Check the period of time for which the blood sample has been stored. Anticoagulated blood using various anticoagulants should be tested within the below mentioned time period (when stored at 2–8°C) • EDTA or heparin—2 days • Sodium oxalate or sodium citrate—14 days • ACD or CPD—28 days • Clotted whole blood—14 days
3. Use of hemolyzed samples	Check the samples before use. Do not use hemolyzed samples
4. Prozoning (zone of antibody excess) or postzoning (zone of antigen excess)	The antigen and the antibody should be present in optimal concentrations of the agglutination to be seen properly. Check the sample volume and the reagent volume used. Both the sample and reagent volume should be equal in slide test and a 5% suspension of cells should be used in tube test. Ensure that there are no air bubbles while dispensing samples and reagents.

Contd.

Possible causes	*Solutions*
5. Outdated or contaminated reagent	Check the expiry date of the reagent. Also check the reagent for contamination. Extreme turbidity may be due to microbial contamination
6. Blocking effect when the cells are coated, e.g. in hemolytic disease of the newborn (HDN), acquired immune hemolytic anemia (IHA)	Check the history of the patient for the presence of disorders in which case the cells may be coated *in vivo*. This can be established by doing a DAT on washed cells.
7. Weak antigens	In the tube test, every tube with negative reaction should be centrifuged and the results should be read again after 5 minutes, so that weak antigens are not overlooked
8. Under centrifugation	Centrifugation should be carried out for 1 minute at 1000 rpm or for 20 seconds at 3400 rpm as per recommended procedures to give enough time for antigen-antibody to bind
9. Vigorous shaking for resuspension of cells after centrifugation	Resuspend the cells slowly and gently after centrifugation. Each laboratory must calibrate its equipment at regular intervals
10. In case of A_1 lectin, A antigen is not fully expressed on the red cells of newborns below one year of age	Check the age of the patient. If below 1 year repeat typing after child not fully expressed on the red reaches 1 year of age

Problem: Hemolysis of Red Blood Cells

Possible causes	*Solutions*
1. Use of wet slides and tubes	Wet glassware can cause hemolysis of RBCs. Ensure that only dry glassware is used for testing

Problem: Weak Agglutination

Possible causes	*Solutions*
1. Preleukemic states and acute myeloblastic leukemia	Check the clinical history of the patient
2. Acquired loss of antigens occur in healthy elderly individuals	Check the age of the patient

Problem: Mixed Field Agglutination in ABO Grouping or Discrepancy Observed Between the Red Cell Group and the Reverse Group

Possible causes	*Solutions*
1. Transfusion of blood of a different group. (e.g. group O to group A or more significantly group A to group O)	It is extremely important to verify the cause of mixed field agglutination. An investigation of the patient's history may help to verify the cause of mixed field agglutination
2. Chimera (this is a condition where an individual possesses more than one population of red cells)	This may be caused by exchange of blood cells during early fetal life of non-identical twins or may be artificially induced by compatible but not same blood group. Check the history of the patient
3. Blood group antigens altered by bacterial enzymes. Some group A individuals with intestinal obstruction, carcinoma of the colon or rectum and other disorders of the lower intestine acquire a B-like antigen	Check the history of the patient for the presence of any of these conditions and perform reverse grouping in case of acquired B to confirm the blood group

Problem: Delayed Agglutination

Possible causes	*Solutions*
1. Subgroups of A, other than A_1 such as A_2, A_3, A_x etc have weakly expressed A antigenic sites on the red cells, hence these red cells give weak reaction with anti-A reagent as compared to A_1 cells	Check these cells with anti A_1 lectin, these cells should not react with anti A_1 lectin. Graded reaction helps the clinician to differentiate between strong (A_1) and other weaker subgroups of A group
2. Reagents used immediately after removing from the refrigerator	The reagent vial must be brought to room temperature prior to starting the test
3. The antigen and antibody are not present in optimal concentrations	The sample volume and the reagent volume dispensed should be as per the instructions given in the protocol

RH TYPING

Problem: False-positive Results

Possible causes	*Solutions*
1. Bacterial contamination (some bacteria will agglutinate all red cell samples thereby giving false-positive results)	Check the reagents for turbidity. Extreme turbidity may indicate bacterial contamination. Such contaminated reagents should not be used for testing
2. The antisera may be contaminated with another antibody. There may have been accidental mixing of antisera; this could be due to interchanging of caps	Check the color of the cap on the antisera bottle. Also check the antisera with the blood of a person whose blood group is known
3. Particles of dust, debris, chemicals or detergents on the slide or in the tube giving nonspecific agglutination	Clean and dry glassware should be used while carrying out the test
4. Peripheral drying or fibrin the test should not be carried out directly under the fan. strands were mistaken for the results should be read within 2 minutes. The vial should be capped agglutination in case of slide test immediately after use	
5. Excessive centrifugation in case of tube test	Ensure that centrifugation is carried out for either one-minute at 1000 rpm or 20 seconds at 3400 rpm. Centrifugation should be adequate to produce a cell button with a clear supernatant but without packing the cells so tightly that they are difficult to dislodge. Each laboratory must calibrate its equipment at regular intervals
6. Rouleaux formation in case of tube test may be mistaken for agglutination. Rouleaux formation is said to occur when the red cells appear like a stack of coins. Rouleaux formation may be caused by the following:	Rouleaux formation can be distinguished from agglutination by adding 2 drops of saline to the reaction mixture; if the clumping of cells dissolves then it indicates rouleaux formation. Check the history of the patient for the conditions like multiple myeloma. The suspension of the cells prepared should be as per the instructions in the package insert

Problem: False-negative Results

Possible causes	*Solutions*
1. Storage of antiseras at higher/lower temperatures than specified	Reagents should be stored at 2–8°C when not in use. Thermal damage due to faulty storage may result in a loss of reactivity. Check the turbidity of the reagents; extreme turbidity may be due to thermal damage. Do not freeze the reagents
2. Blood sample stored for too long than recommended	Check the period of time for which the blood sample has been stored. Anticoagulated blood using various anticoagulants should be tested within the below mentioned time period (when stored at 2–8°C) • EDTA or heparin—2 days • Sodium oxalate or sodium citrate—14 days • ACD or CPD—28 days • Clotted whole blood—14 days
3. Use of hemolyzed samples	Check the samples before use. Do not use hemolyzed samples
4. Prozoning (zone of antibody excess) or postzoning (zone of antigen excess)	The antigen and the antibody should be present in optimal concentrations of the agglutination to be seen properly. Check the sample volume and the reagent volume used. Both the sample and reagent volume should be equal in slide test and a 5% suspension of cells should be used in tube test. Ensure that there are no air bubbles while dispensing samples and reagents
5. Outdated or contaminated reagent.	Check the expiry date of the reagent. Also check the reagent for contamination. Extreme turbidity may be due to microbial contamination
6. Blocking effect when the cells are coated e.g. in hemolytic disease of the newborn (HDN), acquired immune hemolytic anemia (IHA)	Check the history of the patient of the presence of disorders in which the cells may be coated
7. Weak antigens	In the tube test, every tube with negative reaction should be centrifuged and results should be read again after 5 minutes, so that weak antigens are not overlooked
8. Under centrifugation	Centrifugation should be carried out for 1 minute at 1000 rpm or for 20 seconds at 3400 rpm
9. Vigorous shaking for resuspension of cells after centrifugation	Resuspend the cells slowly and gently after centrifugation. Each laboratory must calibrate its equipment at regular intervals

Problem: Hemolysis of Red Blood Cells

Possible causes	*Solutions*
1. Use of wet slides and tubes	Wet glassware can cause hemolysis of RBCs. Ensure that only dry glassware is used for testing

Problem: Delayed or Weak Agglutination

Possible causes	*Solutions*
1. Reagents used immediately after removing from the refrigerator	The reagent vial must be brought to room temperature prior to starting the test. Anti-D (IgG) type reacts at 37°C hence at low temperatures it may not react properly
2. The antigen and antibody are not present in optimal concentrations	The sample volume and the reagent volume dispensed should be as per the instructions given in the protocol
3. In case of weak D or partial D cells in slide test	Should be confirmed by tube test or coombs test

GENERAL INSTRUCTIONS FOR ANTIHUMAN GLOBULIN (COOMBS' REAGENT)

Sources of Error in Antiglobulin Testing—Coombs Cells

False-negative Results

- ***Neutralization of Antihuman Globulin (AHG) Reagent***
  1. Failure to wash cells adequately to remove all serum/plasma. Fill tube at least ¾ full of saline for each wash. Check dispense volume of automated washers.
  2. If increased serum volumes are used, routine wash may be inadequate. Wash additional times or remove serum prior to washing.
  3. Contamination of AHG by extraneous protein. Do not use finger or hand to cover tube. Contaminated droppers or wrong reagent dropper can neutralize entire bottle of AHG.
  4. High concentration of IgG paraproteins in test serum; protein may remain even after multiple washes.
- ***Interruption in testing***
  1. Bound IgG may dissociate from red cells and either leave too little IgG to detect or may neutralize AHG reagent.
  2. Agglutination of IgG-coated cells will weaken. Centrifuge and read immediately.
- ***Improper reagent storage***
  1. AHG reagent may lose reactivity if frozen. Reagent may become bacterially contaminated.
  2. Excess heat or repeated freezing/thawing may cause loss of reactivity of test serum.
  3. Reagent red cells may lose antigen strength on storage. Other subtle cell changes may cause loss of reactivity.
- ***Improper procedures***
  1. Overcentrifugation may pack cells so tightly that agitation required to resuspend cells breaks up agglutinates. Undercentrifugation may not be optimal for agglutination.
  2. Failure to add test serum, enhancement medium or AHG may cause negative test.
  3. Too heavy a red cell concentration may mask weak agglutination. Too light suspension may be difficult to read.
  4. Improper/insufficient serum: cell ratios.
- ***Complement***
  1. Rare antibodies may only be detected when polyspecific AHG is used and active complement is present.

- *Saline*
  1. Low pH of saline solution can decrease sensitivity. Optimal saline wash solution for most antibodies is pH 7.0–7.2.
  2. Some antibodies may require saline to be at specific temperature to retain antibody on cell. Use 37°C or 4°C saline.

False-positive Results

- *Cells agglutinated prior to washing*
  1. If potent agglutinins are present, agglutinates may not disperse during washing. Observe cells prior to addition of antihuman globulin (AHG) or use control tube substituting saline for AHG; reactivity prior to addition of AHG or in saline control invalidates AHG reading.
- *Particles or contaminants*
  1. Dust or dirt in glassware may cause clumping (not agglutination) of red cells. Fibrin or precipitates in test serum may similarly produce cell clumps that mimic agglutination.
- *Improper Procedures*
  1. Over-centrifugation may pack cells so tightly that they do not easily disperse and appear positive.
  2. Centrifugation of test with polyethylene glycol or positively charged polymers prior to washing may create clumps that do not disperse.
- *Cells have positive direct antiglobulin test (DAT)*
  1. Cells that are positive by DAT will also be positive in any indirect antiglobulin test.
- *Complement*
  1. Complement components, primarily C4 may bind to cells from clots or from CPDA-1 donor segments during storage at 4°C and occasionally at higher temperatures. For DATs, use red cells anticoagulated with EDTA, ACD or CPD.
  2. Samples collected in tubes containing silicone gel may have spurious complement attachment.
  3. Complement may attach to cells in specimens collected from infusion lines used to administer dextrose-containing solutions. Strongest reactions are seen when large-bore needles are used to when sample volume is less than 5 µL.

ANTIHUMAN GLOBULIN (AHG OR COOMBS' REAGENT)

Problem: False-positive Results

Possible causes	*Solutions*
1. Presence of colloidal silica, which is absorbed by the red cells when saline is stored in glass bottles	Ensure that all glassware used is clean and dry and properly stored saline is being used for the test. Use freshly prepared normal saline
2. Red cells may be agglutinated before the washing is carried out	Check properly for agglutination before proceeding to the antiglobulin phase
3. Overcentrifugation causes tight packing of the cells that cannot be dispersed easily and is mistaken for a positive control	Ensure that centrifugation is carried out at the proper speed for the appropriate time as per the instructions given in the package insert. Each laboratory must calibrate its equipment at regular intervals

Contd.

Possible causes	*Solutions*
4. Absorption of normal cold antibody and complement onto the cells during the refrigeration of clotted blood sample can give false-positive results. Anticoagulants have anticomplement activity. Refrigerated ACD blood also gives false-positive results at times	The blood sample should be tested as soon as possible after collection and should not be stored. For the indirect antiglobulin test, serum not more than 48 hours old should not be used
5. Use of various drugs and certain disease conditions such as megaloblastic anemia are known to be associated with positive direct antiglobulin test	Check the patient's history for disease conditions like megaloblastic anemia
6. In diseases such as pernicious anemia and multiple myelomatosis, autoagglutination takes place (all erythrocytes are agglutinated non-specifically)	Check the patient's history for the presence of such disorders
7. False-positive Coombs' test is seen in blood with high reticulocyte count	Check the history of the patient for high reticulocyte count

Problem: False-negative Results

Possible causes	*Solutions*
1. Insufficient washing of the sample may lead to neutralization of antihuman globulin by the globulin fraction of the serum	Washing must be carried out thoroughly and for the number of times as mentioned in the pack insert to ensure complete removal of free IgG from the sample
2. Serum residues remaining in the poorly washed glassware can cause neutralization of antihuman globulin	Only clean and dry glassware must be used for testing
3. Anti human globulin reagent not working	To all negative test results, after the antiglobulin phase Coombs control cells should be added. If the Coombs control cells do not agglutinate then the test should be repeated using fresh antihuman globulin
4. Contaminated bovine serum albumin may inactivate antihuman globulin	Ensure that the bovine serum albumin, saline and glassware are free from contamination
5. Antihuman globulin may be neutralized with human globulin	Check the anti-IgG of the human globulin reagent using Coomb's control cells
6. Interrupted or delayed testing. Too much time has elapsed between washing the erythrocytes andadding antihuman globulin reagent, so that antibodies have eluted. The Coombs serum has in fact reacted with the antibody but agglutination is not visible, since the antibodies are no longer bound to the erythrocytes	The washing should be undertaken as quickly as possible to minimize the elution of antibody from the cells. Addition of the antihuman globulin followed by centrifugation and reading of results should be in immediate succession
7. If plasma is used in the indirect antiglobulin test, the complement dependent antibodies may not be detected due to the absence of calcium	Do not use plasma. Serum not more than 48 hours old should be used for the indirect antiglobulin test

Contd.

Possible causes	*Solutions*
8. Improper centrifugation	Under centrifugation leads to false-negative results. Resuspension of centrifuged cells vigorously breaks up weak agglutination leading to false-negative results. Centrifugation should be carried out at the appropriate speed for an amount of time as given in package insert
9. If the red cells are few the reaction is difficult to read	Ensure that 5% suspension of red cells is used in the tests as per the instructions given in the package insert
10. Omission of anti-human globulin in the test by mistake	Ensure that all the reagents are added properly as per the instructions given in the pack insert. Eryclone AHG offers the advantage of being color coded so that it helps in identification
11. The incubation temperature was not the optimal one for the antibodies. It was either too high or too low, so that the antibody coating of the ery-throcytes did not occur	Incubate at the optimal temperatures as per the instructions given in the pack insert
12. Only one drop of antihuman globulin reagent may have been used for the D^u test. If one drop is used then residual anti-D from previous incubation or excess wash solution may neutralize/ dilute the reagent affecting its reactivity and giving rise to false-negative reactions	Ensure that two drop of antihuman globulin reagent are used for the D^u test
13. Antihuman globulin reagent has deteriorated	Confirm the results using Coombs control cells
14. Cord cells sensitized heavily with anti-D yield a false-positive result in direct antiglobulin test	Check the cells for sensitization before testing

CHAPTER

26

COVID and Hematological Changes

HEMATOLOGICAL CHANGES IN NONSEVERE CONDITIONS

Changes in WBC Count and Morphology

The majority of early published laboratory findings of nonsevere coronavirus disease-2019 (COVID-19) infection are collected from hospital admitted patients at a single time point which limits our understanding of the dynamic hematological changes during the course of the disease. Asymptomatic patients constitute at least 20% of the cases; however, available literature is more concerned with the prevalence and transmission risk they carry and overlooking hematological changes. This probably because those patients were sent for self-isolation and further investigation was not recommended particularly with the overwhelmed health systems.

Studies which have looked into the WBC changes in hospitalized COVID-19 patients with none severe symptoms reported a total WBC count range of 3.1–7.6 × 10/L with a mean of 4.3–5.7 × 10/L. Lymphocytopenia was reported in 80.4% of nonsevere patients; however, mean lymphocyte counts was more 1.0 × 10/L in most of the cases. Leukopenia reported in 28.1% of this category with mean neutrophil count reported by different studies to be 0.4–6.6 × 10 /L. It is worth noting that both lymphocytopenia and neutropenia in this group of patients were less prominent than those seen in severe cases.

A case–control study comparing the complete blood count (CBC) of symptomatic COVID-19 confirmed patients with those of symptomatic COVID-19 negative proposed eosinopenia as a potential predicting parameter of COVID-19 rather than lymphopenia and leukopenia.

Changes in RBC Indices

Hemoglobin and to less extent hematocrit are the only RBC indices reported in COVID-19 studies. Available literature showed no significant alteration in hemoglobin of those with a mild/moderate disease.

Changes in Coagulation Indices

Early studies in Wuhan and Germany showed no significant differences in the platelet count of none severe compared with severe COVID-19 patients. A larger study included 926 nonsevere cases reported a mean platelet count of 172,000 (139,000–212,000), which was significantly higher than that reported in severely ill patients. There was no significant changes in the levels of other blood coagulation parameters in nonsevere illness.

HEMATOLOGICAL CHANGES IN SEVERE CONDITIONS

Changes in WBC Count and Morphology

There are consistent data indicating that lymphocytopenia and neutrophilia are features of severe COVID-19 illness.

Risk factors associating with acute respiratory distress syndrome (ARDS) development and death, concluded that increased neutrophils and decreased lymphocytes were risk factors for ARDS, and increased neutrophils is associated with higher risk of death.

Studies addressing peripheral blood film of a COVID-19 patient described a characteristic neutrophil nuclei with C-shaped, single-lobed nuclei, morphologic abnormalities in the granulocytic series and leukoerythroblastic reaction. Blue–green leukocyte inclusions were reported in the circulating neutrophils and/or monocytes of critically ill patients with liver impairment and lactic acidosis and proposed as a poor prognostic indicator. By contrast, activated monocytes were observed in clinically improved patients.

Flow cytometry performed on peripheral blood lymphocytes of ICU patients showed a significant reduction in the absolute T-cell count in most cases as well as $CD45^+$, $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, and $CD16/56^+$ counts. However, unlike immunodeficiency virus (HIV) and cytomegalovirus, the CD4/CD8 ratio was not inverted in all groups of patients.

Many studies have suggested neutrophil-to-lymphocyte ratio (NLR) as an independent risk factor for mortality in severely ill patients with COVID-19 with a cutoff value varied between 3.0 and 13.

Changes in RBC Indices

Meta-analysis results regarding hemoglobin level in severely ill COVID-19 patients are conflicting. A meta-analysis study including 224 severely ill COVID-19 patients reported significantly lower hemoglobin than none severe cases with a weighted mean difference (WMD) of −7.1 g/L; 95% CI, −8.3 to −5.9 g/L. Other study found the difference was not significant. The lowest hemoglobin level was seen in patients who reached a composite endpoint (included admission to ICU, requirement of invasive ventilation, and death). The reduction in Hb may indicate disease progression; however, age and comorbidities are confounding factors in this patient group, and data should be interpreted cautiously.

Elevated red blood cell distribution width (RDW), a component of complete blood counts that reflects RBC variation in volume and size, has been shown to be associated with elevated mortality risk for patients with COVID-19.

Changes in Coagulation Indices

Altered coagulability is a poor prognostic indicator in severely and critically ill patients with COVID-19. The laboratory manifestations of coagulopathy are elevated D-dimer, slight decrease in platelet count, prolonged PT, and reduced fibrinogen level. Thrombotic events and bleeding often occur in subjects with weak constitutions, multiple risk factors, and comorbidities.

Coagulopathy in COVID-19 initiated by endothelial injury results in thrombin generation and fibrinolysis shut down, contributing to a hypercoagulable state, which causes a prolonged PT and aPTT. In the late stages of the DIC, PT, aPTT, fibrinogen, and platelets are decreased due to consumptive coagulopathy as observed in nonsurvivors. Moreover, COVID-19 patients presenting with cardiac injury and elevated troponin-T levels were more prone to coagulation disorders compared with those without cardiac involvement. Studies have also detected lupus anticoagulant and antiphospholipid antibodies in COVID-19 patients, which could contribute to the hypercoagulable state.

Altered coagulability is complicated by venous thromboembolism in 24%, deep venous thrombosis 7%, and pulmonary embolism 19% of severely ill COVID-19 patients. Independent predictive parameters for thromboembolism were pneumonia, old age, spontaneous prolongation of PT >3 s, and aPTT >5 s. Arterial embolism in the forms of acute MI, acute limb ischemia, and storks was also reported in COVID-19 patients.

The contribution of von Willebrand factor (vWF) in thromboinflammation is well established. Several mechanisms are involved. These mechanisms starts with endothelial dysfunction. In severely ill COVID-19 patient, marked increase in vWF and factor VIIIc level was observed similar to that seen in severely septic non-COVID-19 ICU patients. With disease progression, and in the absence of anticoagulant treatment, VWF and fibrinogen levels decline with persistent high D-dimer levels and even higher P-selectin levels indicating poor prognosis.

CHAPTER

27

Immunoassay: Instrumentation, Automation and Point-of-care Testing

INTRODUCTION

An immunoassay is a biochemical test that measures the presence or concentration of a macromolecule or a small molecule in a solution using an antibody (usually) or an antigen (sometimes). The molecule detected by the immunoassay is often referred to as an "analyte". Immunoassay techniques were used traditionally for the assay of polypeptide, thyroid, and steroid hormones in human serum, contributing most to studies in endocrinology. Increasingly, immunoassays have come to contribute to other areas of clinical biochemistry.

There are mainly two types of immunoassays: Qualitative and quantitative.

Difference between Qualitative and Quantitative Immunoassays

Assays which detect the presence or absence of analyte in the sample are known as **Qualitative immunoassays,** e.g., Qualisa Ds-DNA, Qualisa CMV IgG, Qualisa HIV 4.0, Qualisa HBsAg.

Assays that measure/quantify, e.g. the concentration of an analyte in a sample are known as **quantitative immunoassays,** e.g., Qualisa 25 OH vitamin D.

Most common forms of immunoassays are:

- ELISA: Enzyme-linked immunosorbent assay
- CLIA: Chemiluminescence immunoassay
- RIA: Radioimmunoassay
- FIA: Fluorescence immunoassay
- ICT: Immunochromatography test

FLUORESCENCE IMMUNOASSAY

Fluorescent immunoassays (FIA) are simply a different type of immunoassay. The key variable is the biochemical technique used for detecting the binding of the "detection" antibody and the analyte molecule. The advantages of a fluorescent detection system have been known for many years. These include higher sensitive detection of the analyte, simplified reagents, and simpler assay designs. Several breakthroughs have occurred over the past few years that have enabled the implementation of rapid fluorescence-based immunoassay systems.

A modern fluorescent-based immunoassay use as the detection reagent a fluorescent compound which absorbs light or energy (excitation energy) at a specific wavelength and then emits light or energy at a different wavelength. The difference between the wavelength of the excitation light and the emission light is called the **Stokes shift.** The greater the shift or difference in the wavelength the less interference there will be by having the excitation light detected as part of the emission light (Fig. 27.1). Recently, several technical

improvements have occurred that has enabled the implementation of a high sensitivity rapid immunoassay systems. These include the availability of narrow wavelength light sources, newer more stable fluorophores that have very wide Stokes shifts, stable solid state light detectors and microprocessors to process and analyse the data from each test.

Fluorescence: The phenomenon in which molecules absorb light at one wavelength and emit light at longer wavelength when it is illuminated by a different wavelength (Figs 27.1 and 27.2).

Fluorophores: The materials having property of emitting light are known as fluorophores.

Recently fluorescence technology has been vastly used in development of Immunoassay field.
- Uses antibody/antigen labeled with a fluorophores

Types of Fluorescence Immunoassays

Presently for diagnostic use two types of fluorescence immunoassays are used: Steady-state fluorescence and time-resolved fluorescence.

Steady-state Fluorescence

A type of spectroscopy where the intensity of the fluorescence emitted by molecules excited by constant illumination of ultraviolet light is detected as a function of wavelength.

The applications of steady-state fluorescence include excitation and emission scans, synchronous scans and maps, steady-state fluorescence anisotropy, excitation-emission maps, kinetic measurements, and temperature maps.

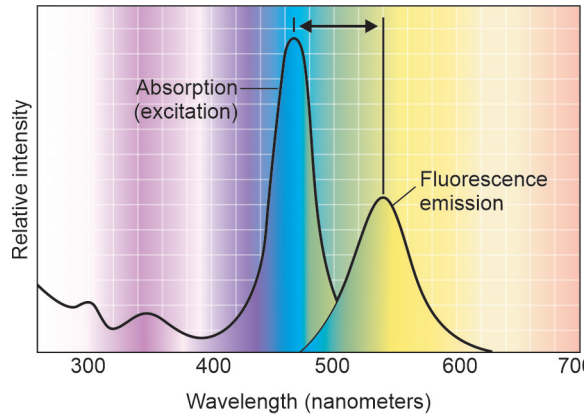


Fig. 27.1: A depiction of relative intensity and wavelength of a fluorescent detection agent: Stoke shift between adsorption and emission light spectra

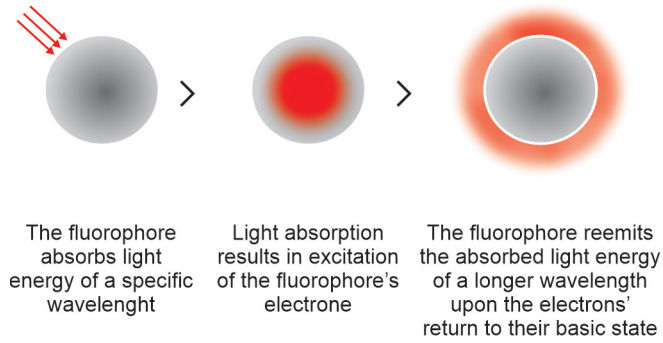

The fluorophore absorbs light energy of a specific wavelenght
Light absorption results in excitation of the fluorophore's electrone
The fluorophore reemits the absorbed light energy of a longer wavelength upon the electrons' return to their basic state

Fig. 27.2: Fluorescence phenomenon

Time-resolved Fluorescence (TRFIA)

Time-resolved fluorescence spectroscopy is a spectroscopy technique used to monitor interactions between molecules and motions that occur in short periods. The ability to measure changes in the picoseconds or nanosecond time range makes it a useful technique in biomolecular structure analysis and dynamics.

Time-resolved fluorescence spectroscopy can detect events within a fluorophore's environment. The events that can be measured are fluorescence decay, indicated by a decrease in fluorescence after excitation, and polarization anisotropic decay, where the reorientation of the emission dipole during excitation is measured.

Measured decay time-resolved fluorescence spectroscopy works based on the duration of the excited state, which can be shortened by certain dynamic events including macromolecular conformation changes, solvent relaxation, side-chain rotation, interactions between neighboring residues, and other changes in the local environment. The variation is of only a few picoseconds or tens of nanoseconds, meaning measurable changes must occur within this time range. Developments in this type of measurement are making it possible to understand structural information, for example, by discerning distances between probes.

Stokes Shift

The difference between the excitation and the emission wavelength is known as stokes shift (Fig. 27.3).

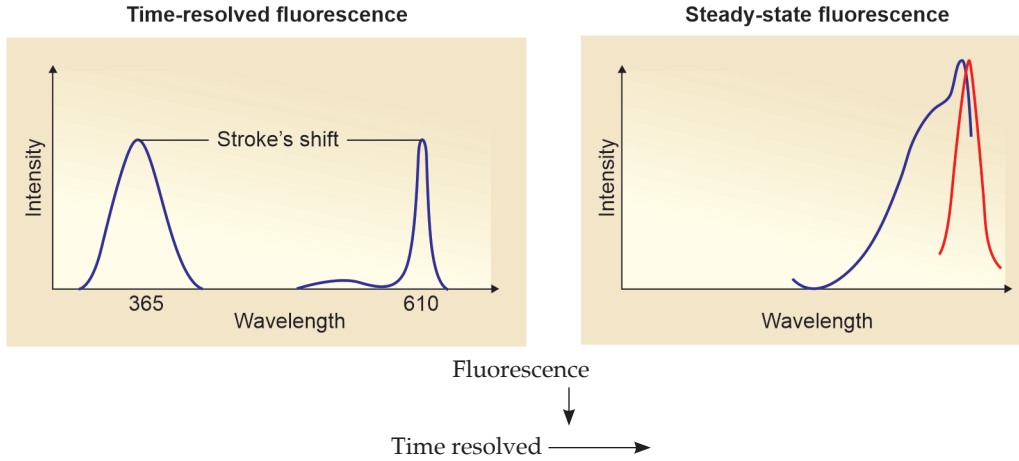


Fig. 27.3: Differences in time-resolved and steady-state fluorescences

Steady State

Steady-state	*Time-resolved*
The light emitted is detected while excitation is still going on	The emitted light is detected after the excitation has taken place
High background signal	Low background signal
Stokes shift is relatively very small which can cause self quenching of reagents	Stokes shift is high
Decay time is very less usually in nanoseconds	Decay time is more usually in micro-mili seconds
Biological samples such as serum/tissue often contain autofluroscence particles that can contribute to background signals leading to background noise	Since dacay time is more, autofluoroscence of samples settles before reading reading is taken

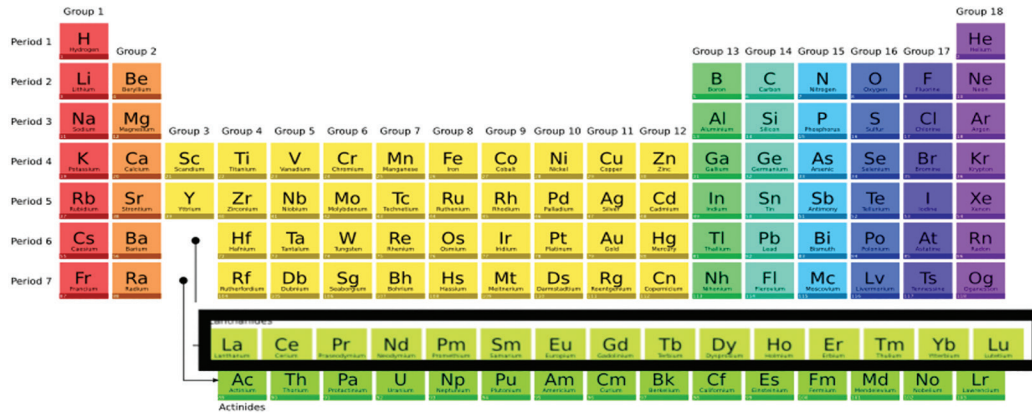


Fig. 27.4: Fluorescent chemical elements

Time-resolved Fluorescence Immunoassay (TRFIA)

To develop time resolved fluorescence immunoassays special types of fluorophores are used, called **lanthanides** or earth materials.

Lanthanides

- They are a group of uniquely fluorescent chemical elements (Fig. 27.4)
- They have very low absorption coefficient and slow emission rates
- This results in prolonged fluorescence decay time (0.1–3) mili seconds
- These elements form trivalent cations (Eu^{3+}) and displays emission in aqueous solution

Point-of-care Testing

Point-of-care testing (POCT), also called near-patient testing or bedside testing, is defined as medical diagnostic testing, at the time and place of patient care. The driving notion behind POCT is to bring the test conveniently and immediately to the patient. This increases the likelihood that the patient, physician, and care team will receive the results quicker, which allows for better and immediate decisions to be made.

The goal is to collect the specimen and obtain the results in a very short period of time at or near the location of the patient so that the treatment plan can be initiated.

The FIAcheck 1.0 portable instrument is coupled with an in-built battery source to provide the system when the primary source of power is down, and an in-built thermal printer which enables the healthcare provider to quickly access patient test results from a POCT instrument.

Instrument: FIAcheck 0.1 Time-resolved Fluorescence Analyzer

Time-resolved fluorescence (TRF) spectroscopy is a technique used to monitor interactions between molecules and motion that occur in the short periods (Fig. 27.5).

During standard fluorometric detection excitation and emission are simultaneous; the light emitted by the sample is measured while excitation is taking place. In contrast to this, TRF relies on the use of very specific fluorescent molecules, called lanthanide chelate labels, which allow detection of the emitted light to take place after excitation has occurred (Fig. 27.6).

Key Specifications of Analyzer

- Easily portable
- 7 inch LCD screen

- RS-232 port for data transfer
- LIS/HIS
- In-built thermal printer
- In-built battery
- USB port for data transfer and software update
- Multiple test items

Fig. 27.5: FIAcheck instrument

Why FIAcheck is Needed?

- Low TAT (turnaround time)
- High accuracy and high sensitivity
- Availability of wide range of parameters
- Smaller pack sizes
- No calibration required thus no addition or hidden cost to user
- Minimum skills required to perform the test

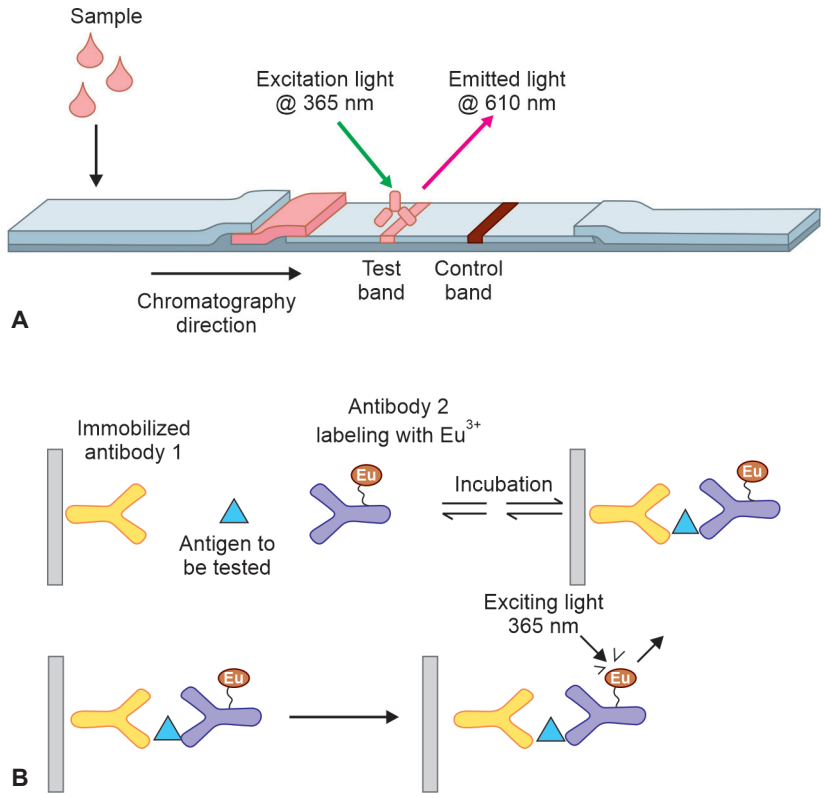


Fig. 27.6: Principles of the FIA test

Two Different Test Principles

1. Double-antigen Sandwich (Example of TSH)

a. Test line coated with immobilized TSH monoclonal antibody 1.
b. Sheep anti-chicken IgY in the control line.
c. Mixture of TSH mouse monoclonal antibody 2 on binding pad along with chicken IgY labeled with fluorescent microspheres.

d. TSH in the samples binds to the fluorescent microspheres labeled with TSH mouse monoclonal antibody 2 in the binding pad.
e. The Ag-Ab-F moves forward due to capillary action.
f. The complex binds to the immobilized antibody on the test area forming complex of fluorescence labeled TSH monoclonal antibody 2-TSH-TSH monoclonal antibody 1
g. Fluorescent labeled chicken IgY binds with goat-anti chicken IgG on control line
h. When test strip is inserted the analyzer scans both T and C and quantify based on the regression graph uploaded by the calibration.

2. Competitive (Example of T3)

a. The test line is coated with T3-BSA full antigen complex.
b. Sheep anti-chicken IgY coated in the control pad.
c. Binding pad coated with T3 sheep monoclonal antibody labeled with fluorescent microspheres and chicken IgY labeled with fluorescent microspheres.
d. T3 in the samples binds to the monoclonal T3 antibody labeled with fluorescent microspheres in the binding pad.
e. Unconjugated fluorescent microspheres with T3 antibody binds to the T3-BSA antigen on the test area forming a band.
f. The chicken IgY labeled with fluorescent microspheres binds to sheep anti-chicken IgY in the control line forming a band.
g. The florescence intensity is inversely proportional to the concentration of T3 in the sample.

Usage

Test procedures are divided into two:

- One step procedure

  Example: FSH test procedure

  1. To calibrate the FIAcheck FSH kit, scan the QR code card provided with the kit.
  2. Remove FIAcheck FSH test device from sealed pouch and place it horizontally on a clean table, label the device with sample identity.
  3. Dispense 100 µL of the sample onto the sample port in the FIAcheck FSH test device.
  4. Incubate at room temperature (18–30°C) for 15 minutes.
  5. After 15 minutes, insert the test device immediately into the FIAcheck analyzer and read results.

- Two steps procedure

  Example: TSH test procedure

  1. To calibrate the FIAcheck TSH kit, scan the QR code card provided with the kit.
  2. Dispense 400 µL of sample diluent into the empty sample dilution vial.
  3. Add 100 µL of the test sample into this sample diluent and mix by rinsing the tip 3 times.
  4. Close the lid of the sample dilution vial, label with sample identity and mix the content of the vial by gently inverting it for 10 times.
  5. Remove FIAcheck TSH test device from sealed pouch and place it horizontally on a clean table, label the device with sample identity.
  6. Dispense 100 µL of the above mixture at the sample port in the FIAcheck TSH test device.
  7. Incubate at room temperature (18–30°C) for 15 minutes.
  8. After 15 minutes, insert the test device immediately into the FIAcheck analyzer and read results.

List of Parameter Menu

Marker	*Product Name*	*Pack size*
Thyroid marker	FIAcheck TSH	25T
	FIAcheck TT3	25T
	FIAcheck TT4	25T
Fertility marker	FIAcheck FSH	25T
	FIAcheck LH	25T
	FIAcheck PRL	25T
	FIAcheck β-hCG	25T
	FIAcheck AMH	10T
Cancer marker	FIAcheck PSA	10T
Rhematology	FIAcheck Anti-CCP	10T
Diabetic marker	FIAcheck HbA1c	25T
Inflammation	FIAcheck CRP	25T
	FIAcheck PCT	10T
	FIAcheck IL-6	10T
Anemic marker	**FIAcheck Ferritin**	**10T**
Coagulation	**FIAcheck D-Dimer**	**10T**
Cardiac markers	FIAcheck cTnI	10T
	FIAcheck NT proBNP	10T
Vitamins	FIAcheck 25-OH-Vitamin D	10T
	FIAcheck B_{12}	10T
Allergy	FIAcheck IgE	10T

Bold texts are hematological parameters

POLYMERASE CHAIN REACTION IN CLINICAL DIAGNOSIS (AS POINT-OF-CARE INVESTIGATION SYSTEM)

What is a PCR?

The polymerase chain reaction (PCR) is a technique discovered by Dr Kary B Mullis in 1985 to synthesize multiple copies of a specific fragment of DNA from a template. The PCR reaction consists of cycling the DNA through three specific temperatures in a buffer solution consisting of the template, primers, nitrogenous bases and enzymes. It is capable of producing enormous amplification (i.e. identical copies) of a short DNA sequence from a single molecule of starter DNA. It is used to amplify a specific DNA (target) sequence lying between known positions (flanks) on a double-stranded (ds) DNA molecule.

The amplification process is mediated by oligonucleotide primers that, typically, are 20–30 nucleotides long. The primers are single stranded (ss) DNA that have sequences complementary to the flanking regions of the target sequence. Primers anneal to the flanking regions by complementary-base pairing (G=C and A=T) using hydrogen bonding.

The amplified product is known as an amplicon.

Generally, PCR amplifies smallish DNA targets 100–1000 base pairs (bp) long. (It is technically difficult to amplify targets >5000 bp long.)

PCR has many applications in research, medicine and forensic science. PCR is a common and most indispensable technique used in medical laboratory and clinical laboratory research for a broad variety of applications including biomedical research and criminal forensics.

Polymerase chain reaction (PCR) is also extensively used in diagnosis of human diseases. Continuous enhancements to this filed have resulted in PCR becoming the gold standard techniques with very high sensitivity and high specificity in the diagnosis of various human diseases caused by:

- Viral, bacterial, fungal and parasitic pathogens
- Inherited disorders or genetic diseases
- Virally induced cancers, leukemia, lymphomas and solid tumours to name a few.

With its ability to detect minute amounts of target DNA or RNA markers contained in tissues or biological fluids, PCR has improved the rapidity and accuracy of diagnosis, enhanced understanding of pathogenesis, and helped identify infectious causes for diseases previously considered idiopathic.

Principle of PCR

A PCR based diagnostic test starts with the process of target selection and primer design-Identifying the sequence to be amplified and designing the primers accordingly. This critical step is responsible for the specificity of the PCR technique. The tremendous growth in genome sequence data coupled with advances in in-silico analysis have made this task comparatively more efficient than earlier. Primers are designed complementary to the template DNA. Two primers are used, one that binds to the sense strand, and another to the antisense strand. The primers are added, along with the DNA polymerase, dNTPs, Magnesium Chloride and DNA template strand.

Almost all the PCR methods are based on thermal cycling wherein the reactants in a PCR are exposed to repeated cycles of heating and cooling to allow different temperature dependent steps to proceed continuosly at the specified temperatures. PCR comprises two main reagents namely primers and DNA polymerase. Primers or oligonucleotides are short single stranded DNA fragments having complementary sequence to that of the target DNA strand. DNA polymerase is an enzyme that helps in the addition and extension of the new DNA strand. Almost all PCR applications employ a heat-stable DNA polymerase, known as Taq polymerase, an enzyme originally isolated from the thermophilic bacterium Thermus aquaticus. This enzyme is a heat stable enzyme. Apart from these two main components PCR also comprises of a PCR buffer, magnesium chloride and dNTPS (deoxyribonucleotide triphosphate).

One PCR cycle consists of three steps:

- Denaturation
- Annealing
- Extension

Denaturation by Heat

Heat (usually >90°C) separates double-stranded DNA into two single strands, referred to as "denaturation". Since the hydrogen bonds linking the bases to one another are weak, they break at high temperatures, whereas the bonds between deoxyribose and phosphates, which are stronger covalent bonds, remain intact.

Annealing Primer Binding to Target

Primers are short, synthetic sequences of single stranded DNA typically consisting of 20–30 bases, with a biotin-labeled 5′ end to aid in detection. They are specific for the target region of the organism. Two primers are included in the PCR, one for each of the complementary single DNA strands that was produced during denaturation. The beginning of the DNA target sequence of interest is marked by the primers that anneal (bind) to the complementary sequence.

Annealing Temperature

Annealing usually takes place between 40°C and 65°C, depending on the length and base sequence of the primers. This allows the primers to anneal to the target sequence with high specificity.

Extension

Once the primers anneal to the complementary DNA sequences, the temperature is raised to approximately 72°C and the enzyme Taq DNA polymerase is used to replicate the DNA strands. Taq DNA polymerase is a recombinant thermostable DNA polymerase from the organism Thermus aquaticus and, unlike normal polymerase enzymes is active at high temperatures.

Taq DNA polymerase, begins the synthesis process at the region marked by the primers. It synthesises new double-stranded DNA molecules, both identical to the original double stranded target DNA region, by facilitating the binding and joining of the complementary nucleotides that are free in solution (dNTPs). Extension always begins at the 3' end of the primer making a double strand out of each of the two single strands. Taq DNA polymerase synthesises exclusively in the 5' to 3' direction. Therefore, free nucleotides in the solution are only added to the 3' end of the primers constructing the complementary strand of the targeted DNA sequence.

Following primer extension, the mixture is heated (again at 90–95ºC) to denature the molecules and separate the strands and the cycle repeated.

Each new strand then acts as a template for the next cycle of synthesis. Thus amplification proceeds at an exponential (logarithmic) rate, i.e. amount of DNA produced doubles at each cycle. 30–35 cycles of amplification can yield around 1 μg DNA of 2000 bp length from 10–6 μg original template DNA. This is a million-fold amplification.

Initially, the 3 different stages at 3 different temperatures were carried out in separate water baths but nowadays, a thermal cycler is used (a machine that automatically changes the temperature at the correct time for each of the stages and can be programmed to carry out a set number of cycles).

Polymerase Chain Reaction

A typical thermal cycle might be as mentioned in Fig. 27.7 and as follows: Heat denaturation at 94ºC for 20 seconds primer annealing at 55ºC for 20 seconds primer extension at 72ºC for 30 seconds Total time for one cycle = approx. 4 minutes.

1. **DNA is denatured:** Primers attach to each strand. A new DNA strand is synthesized behind primers on each template strand.
2. **Another round:** DNA is denatured, primers are attached, and the number of DNA strands are doubled.
3. **Another round:** DNA is denatured, primers are attached, and the number of DNA strands are doubled.

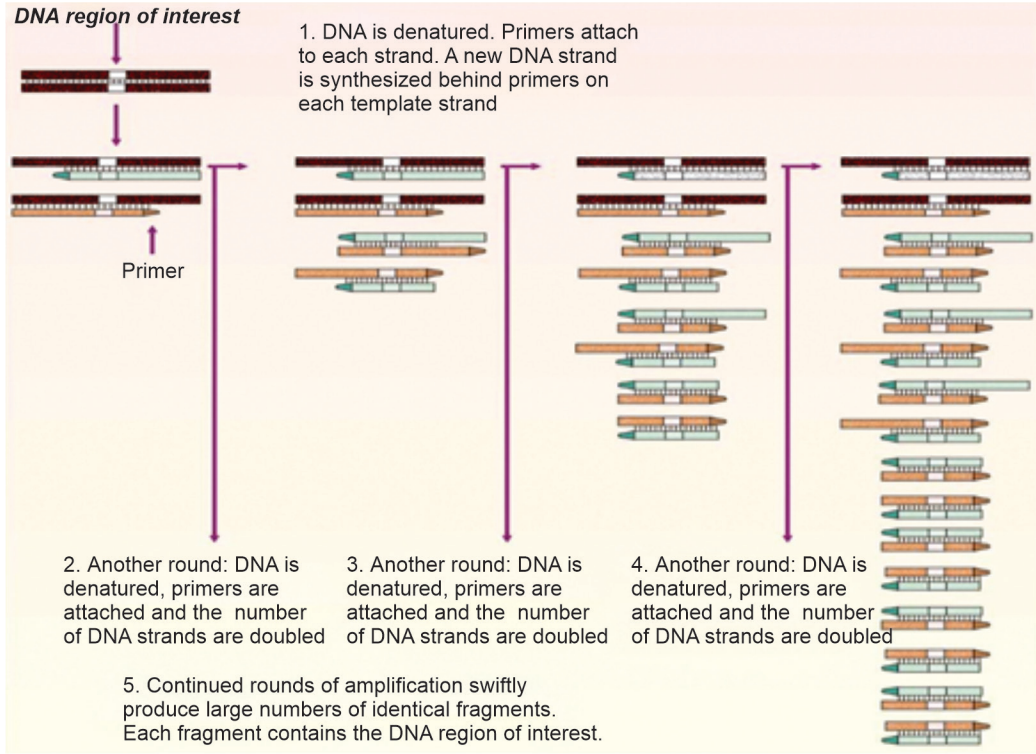

DNA region of interest
1. DNA is denatured. Primers attach to each strand. A new DNA strand is synthesized behind primers on each template strand
Primer
2. Another round: DNA is denatured, primers are attached and the number of DNA strands are doubled
3. Another round: DNA is denatured, primers are attached and the number of DNA strands are doubled
4. Another round: DNA is denatured, primers are attached and the number of DNA strands are doubled
5. Continued rounds of amplification swiftly produce large numbers of identical fragments. Each fragment contains the DNA region of interest.

Fig. 27.7: A typical thermal cycle in PCR

4. **Another round:** DNA is denatured, primers are attached, and the number of DNA strands are doubled.
5. **Continued rounds:** Continued rounds of amplification swiftly produce large numbers of identical fragments. Each fragment contains the DNA region of interest.

Types of PCR

RT-PCR

This is reverse transcriptase-PCR and is a two-stage procedure used for the amplification of RNA. The first stage employs an enzyme called reverse transcriptase, which synthesizes a DNA strand complementary to the RNA of interest by using one of the PCR primer as its primer. The complementary DNA is then used in the second stage as the starting material for PCR amplification by a conventional thermostable DNA polymerase.

Nested PCR

It is a PCR done in two steps, a primary PCR reaction and a nested reaction. The primary (or first) reaction uses a set of primers to generate a product that serves as the template for the nested (or second) reaction. The nested reaction uses a set of PCR primers specific for a region within the amplified product from the first reaction. Therefore, the nested reaction often serves as a confirmation for the specificity of the PCR products amplified in the primary reaction.

Real-time PCR

Combines PCR amplification and detection into a single step. The basic principle of real-time quantitative PCR is the detection of target sequences using a fluorogenic 5′ nuclease assay (often called 'TaqMan').

Real-time or quantitative PCR (qPCR) is used for the amplification of DNA in a linear manner in order to quantify absolute or relative amounts of target sequence in a sample. With the help of a fluorescent reporter, the amount of generated DNA can be measured. In qPCR, DNA amplification is monitored at each cycle of PCR.When the DNA is getting amplified logarithmically at each cycle, the amount of fluorescence increases over the basal level. The thermal cycle at which the signal exceeds the fluorescence detection threshold is known as the threshold cycle (C_T) or crossing point. A standard curve of log concentration against C_T can be made by making use of multiple dilutions of a known amount of standard DNA. The quantity of DNA or cDNA in an unknown sample can thus be determined from its C_T value (Fig. 27.8).

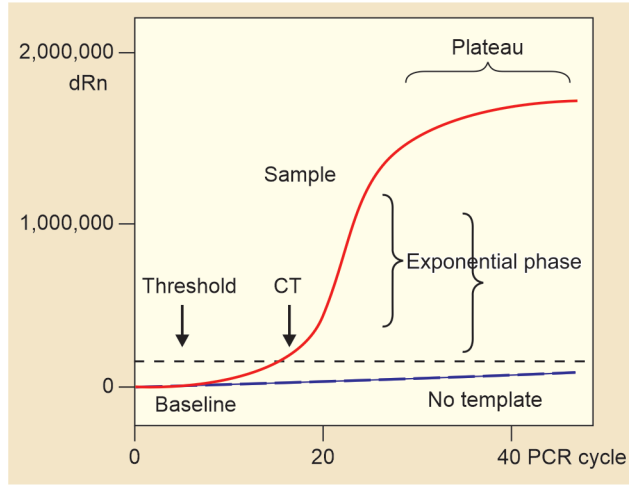


Fig. 27.8: Depiction of real-time PCR plot *[Courtesy:* National Programme on Technology-enhanced Learning (NPTEL)]

Different Chemistries are Used for Detection by q PCR

a. Use of an intercalating dye like the SYBR® Green I dye which incorporates between the base pairs of DNA (Fig. 27.9). This detection method is suitable when the PCR reaction

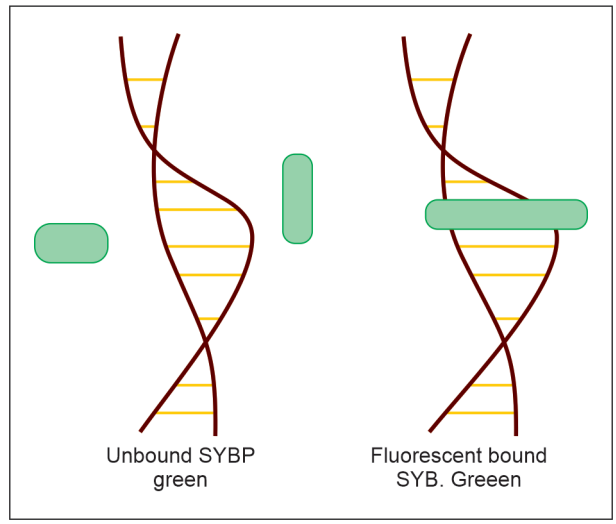


Fig. 27.9: Mechanism of attachment of SYBR green dye to DNA [*Courtesy:* National Programme on Technology-enhanced Learning (NPTEL)]

generates a specific product, as the dye is capable of intercalating into any double-stranded DNA product.

b. Use of primer or short oligonucleotide specific to the target of interest, as in TaqMan® probes, molecular Beacons™, or scorpion primers. In case of molecular beacons, they are labeled with a fluorescent dye or quencher and do not exhibit any significant fluorescence in the free, unhybridized condition. But upon binding to the template, the probe becomes fluorescent as the quencher gets distanced from the fluorescent reporter. The amount of PCR product amplified is directly proportional to the amount of fluorescence (Fig. 27.10).

c. Taqman probe-based detection: While in the case of TaqMan® probes, fluorescence occurs when the dye is clipped from the probe during the polymerase extension (Fig. 27.11).

Uses of PCR

1. **Ligation-mediated PCR:** It uses small DNA oligonucleotide 'linkers' (or adaptors) that are first ligated to fragments of the target DNA.
2. **Methylation-specific PCR (MSP):** It is used to identify patterns of DNA methylation at cytosine-guanine (CpG) islands in genomic DNA.
3. **Multiplex-PCR:** It uses several pairs of primers annealing to different target sequences.
4. **Variable number of tandem repeats (VNTR) PCR:** It targets areas of the genome that exhibit length variation.
5. **Asymmetric PCR:** It preferentially amplifies one strand of the target DNA.
6. **Nested PCR:** It is used to increase the specificity of DNA amplification. Two sets of primers are used in two successive reactions. In the first PCR, one pair of primers is used to generate DNA products, which may contain products amplified from nontarget areas. The products from the first PCR are then used as template in a second PCR, using one ('hemi-nesting') or two different primers whose binding sites are located (nested) within the first set, thus increasing specificity.

The advantages of this system include high reproducibility, the capability of handling large numbers of samples, the potential for quantitative results, and decreased turnaround time. The disadvantages include high instrument cost and the requirement for technical proficiency.

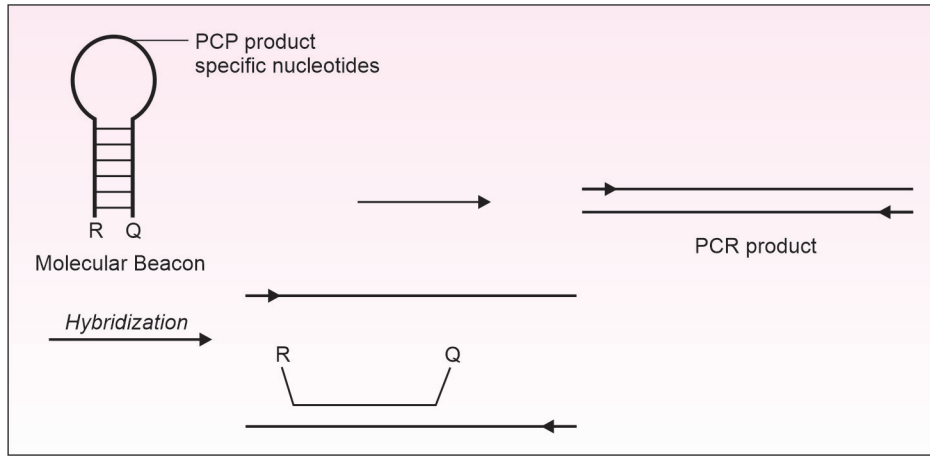


Fig. 27.10. Molecular Beacon-based PCR detection [*Courtesy:* National Programme on Technology-enhanced Learning (NPTEL)]

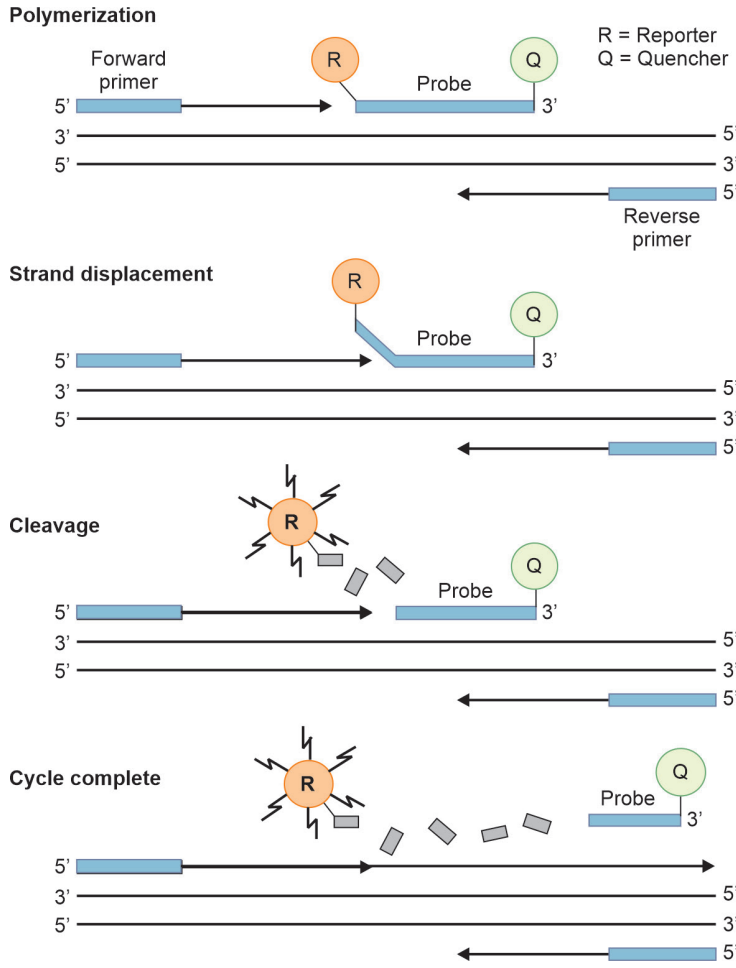


Fig. 27.11. Taqman probe-based PCR detection (*Courtesy:* Wikipedia)

Multiplex PCR

It is a PCR designed to detect more than one target sequence in a single PCR reaction. The assay uses two or more sets of primers. Each set of primers is specific for a different target sequence. The assay is most commonly used for simultaneous detection of multiple viral genes and differentiation of genotypes or subtypes of related microorganisms.

Differential PCR

Differential PCR can sometimes be used to distinguish closely related targets. Differential PCR is done either in a multiplex format using two or more sets of primers or by running two separate PCR assays.

Applications of PCR

PCR technique has vast applications. It can be used for DNA cloning application for sequencing, gene cloning and manipulation, gene mutagenesis; construction of DNA-based phylogenies, functional analysis of genes, diagnosis and monitoring of hereditary diseases, amplification of ancient DNA, analysis of genetic fingerprints for DNA profiling (for example, in forensic science and parentage testing), and finally it has a huge impact in the detection of pathogens for infectious disease diagnosis.

Advantages of PCR over Conventional Techniques

a. Quick, accurate, more sensitive and specific
b. Has fast turnaround time as compared to other tests such as culture
c. Has more wider application such as detecting infectious diseases, genetic testing, forensics, drug resistance, and tumor marker detection and monitoring.

Limitations/Difficulties

Technique-related Limitations

While a very powerful technique, PCR can also be very tricky. The polymerase reaction is very sensitive to the levels of divalent cations (especially $Mg2^{+}$) and nucleotides, and the conditions for each particular application must be worked out. Primer design is extremely important for effective amplification. The primers for the reaction must be very specific for the template to be amplified. Cross reactivity with non-target DNA sequences results in nonspecific amplification of DNA. Also, the primers must not be capable of annealing to themselves or each other, as this will result in the very efficient amplification of short nonsense DNAs. The reaction is limited in the size of the DNAs to be amplified (i.e., the distance apart that the primers can be placed). The most efficient amplification is in the 300–1000 bp range, however, amplification of products up to 4 Kb has been reported. Also, Taq polymerase has been reported to make frequent mismatch mistakes when incorporating new bases into a strand.

PCR reagents are very sensitive to heat and are stored at temperatures between –20°C and –80°C and cannot be subjected to multiple freeze thaw cycles. Laboratories running PCR are typically equipped with good sample storage facilities and skilled technicians.

The most important consideration in PCR is contamination. If the sample that is being tested has even the smallest contamination with DNA from the target, the reaction could amplify this DNA and report a falsely-positive identification. For example, if a technician in a crime lab sets up a test reaction (with blood from the crime scene) after setting up a positive control reaction (with blood from the suspect) cross contamination between the samples could result in an erroneous incrimination, even if the technician changed pipette tips between samples. A few blood cells could volatilize in the pipette, stick to the plastic of the pipette, and then get ejected into the test sample. The powerful amplification of PCR may be able to detect this cross contamination of samples. Modern labs take account of this fact and devote tremendous effort to avoiding this problem, while strictly following Good Laboratory Practices to ensure accurate reporting of results.

Because of the high capex and opex involved in the above, clinical diagnostic applications of PCR have been highly centralized and limited to large hospitals and laboratories having specialized infrastructure and resources. Samples hence have to be transported to these centralized facilities, maintaining a cold chain. These and the need for batch testing often lead to long turnaround time for results thus severely limiting the clinical value of the test.

New innovations in recent times have come up with solutions that enable decentralization of PCR testing by reducing/eliminating dependence on laboratory infrastructure and skilled man power and bringing PCR testing closer to the patient.

Point-of-care PCR for Clinical Diagnosis

In order to cater to the need for PCR based diagnosis in distributed settings, PCR devices are becoming smaller, simpler, more accurate, and easy to handle. The introduction of GeneXpert by cepheid, Sunnyvale, california paved the way for more affordable and less

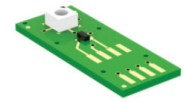
complicated real time PCR testing. However, infrastructure dependence resulted in this platform being restricted to district level settings.

Recently, Molbio Diagnostics, Goa, India has introduced Truenat, a portable, battery operated, real time PCR platform for point of care use that enables the end user to perform molecular tests at the first point of patient contact even in primary health settings. With ready to use, room temperature stable reagents, multi-disease testing capability and turn around time of 1 hour from sample to result, truenat has brought in a paradigm shift in PCR testing by removing all the bottlenecks of a conventional PCR and making it accessible and affordable at all levels of the health care chain.

TRUENAT PLATFORM

Truenat test for MTB and Rif resistence has been endorsed by WHO and recommended for use as the initial test for TB as a replacement to smear microscopy.

Truenat test for Covid-19 is endorsed by ICMR and has played a major role in RTPCR testing during the pandemic (Figs. 27.12 and 27.13).

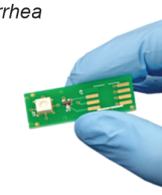


Trueprep™ Auto universal sample prep	Trueprep™ Auto Prep at point of care	Truenat™ Microchip	Truelab™ Uno, Duo and quattro
• Automated sample processing • PCR ready DNA/RNA in 20 minutes • Inactivate virus at point of collection		• Micro Smart thermal cycler • Smart memory chip • Self identification • Standard curve for viral load quantization, etc.	• Network data transfer ability and automated reporting • Real time, quantitative micro PCR portable 8 hours battery backup 3 channel fluorescence • DUO-2 Bays, Random access [–20 runs per day]; Quattro - 4bays, Random access [40 runs per day]

Fig. 27.12: Truenat platforms

Products available

- MTB screening CE
- MTB-RIF CE
- HBV with VL
- HIV-1 with EID+VL
- HCV with VL
- H1N1 CE
- Dengue CE
- HAV CE
- SARS CoV-2 CE
- Chikungunya CE
- Dengue/Chikangunya CE
- Malaria Pf CE
- Malaria Pv/Pf CE
- *Salmonella* CE
- *Chlamydia*
- HLA-B27 CE
- HEV CE
- COVID-19 CE
- Gonorrhea CE
- *Chlamydia/Gonorrhea*
- *Trichomonas* CE
- Rabies CE
- HPV-*HR* CE
- Influenza A/B CE
- GBS CE
- Beta CoV CE
- *Leptospira* CE



Products coming soon

- SA/MRSA
- H3N2/H1N1
- ZIKA
- *Staphylococcus aureus* (SA)
- MTB/NTM
- MTB-INH
- MTB-BDQ
- ***Neisseria meningitidis*** (Nm)
- SA/PA
- *Hemophillus influenzae* B (HIB)
- *Shigella*
- *Leishmania*
- MTB-FQ
- Cholera
- *Pseudomonas aeruginosa* (PA)
- *Streptococcus pneumoniae* (SP)
- Nipah
- Sickle Cell Anemia screeninig
- Kysanur Forest Disease (KFD)
- *Mycoplasma*

Products PIPELINE

- *Klebsiella*
- HIV-2
- CMV
- EBV
- HSV (1/2)
- HHV-6 (A/B)
- Yellow fever virus
- BCR-ABL for chronic myeloid leukemia
- Rubella
- Rubeola
- Rubula
- H5N1
- Japanese encephalitis virus (JEV)
- Measles
- *Bordetella pertussis*
- *Toxoplasma gondii*
- Rotavirus
- Syphilis
- Mumps
- MERS (Middle East Respiratory syndrome)
- *Clostrdium difficile*

Fig. 27.13: Truent™ real-time micro-PCR chips. Note: Texts in bold and red are haematology related parameters

AUTOMATION IN HEALTH CARE

Automation in healthcare, particularly, medical diagnostics is relatively new. It has taken a cue from the manufacturing industry and has been proved extremely successful. Automation has become necessary when large numbers of specimens are processed every day. The increased demand for better services and the expectation of quick results by the patients mean the laboratories need to look out for ways to be efficient.

Laboratory and Blood Bank Automation

Laboratory automation comprises many different automated instruments, devices, software algorithms, and methodologies used to enable, expedite and increase the efficiency and effectiveness of the laboratory operations. For any laboratory with high sample load, it is inconceivable without automation to achieve timely progress and turn-around times (TATs).

The functional steps in automation include sample labelling with barcodes, transportation, centrifugation and pre-analytical processing. Automation also continues to the analytical and post-analytical processes as well.

There have been rapid technological advances in blood banking in South Asian region over the past decade with an increasing emphasis on quality and safety of blood products. The conventional technique has given way to newer techniques such as column agglutination technology (CAT), chemiluminescence immunoassay (CLIA) and enzyme linked immunosorbent assay.

Large blood banks have a heavy workload and therefore there is a risk of errors when operation is manual which compromises blood safety. Often it is difficult to have a skilled staff. Automation eliminates manual errors, reduces manpower requirements and has uniform performance.

1. Immunohematology Systems

Tulip introduces matrix autoMax-80-Automation with maximum benefits based on column agglutination technique for blood group screening under immunohematology. It is modular dual need system to ensuring safe blood and safe blood transfusion (Fig. 27.14). Following benefits with matrix AutoMax-80

- Modular system
- Integrated barcode system
- Intelligent piercing module
- Intelligent software
- On-board inventory.

Fig. 27.14: Matrix autoMax-80

2. Automated Serological Tests for Transfusion Transmissible Infections

Automated systems generally offer substantial advantages in terms of quality, reproducibility and reduce operational errors. All enzyme linked immunosorbent assay (ELISA) systems employed by the blood bank need a basic level of automation like automated plate washers and readers. Many fully automatic walk away systems

are available in the market which can perform several types of ELISA and have an open platform.Tulip introduces euphoreia 4.1- Fully automated ELISA processor for processing up to 4 microplates of ELISA.

Various automated chemiluminescence immunoassay analyzers are now commercially available and are replacing conventional enzyme immunoassays. Tulip also offers Electra-FA - Fully automated Double channel Micro well CLIA analyser with high speed (Fig. 27.15). Tulip provides full parameters i.e. HIV, HCV, HBsAg, syphilis Ab and malaria kits for transfusion transmissible infections testing on CLIA and ELISA platform.

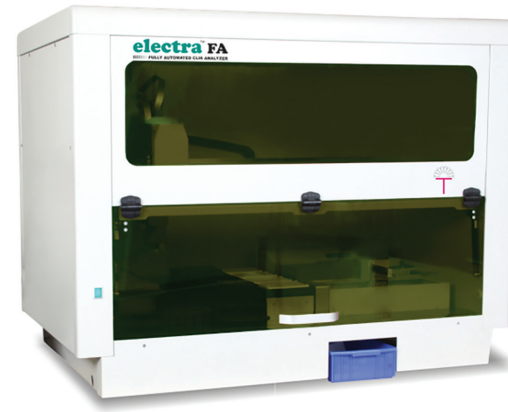


Fig. 27.15: Electra FA

Rationale for Automation

It is primarily a means to improve efficiency and reduce human errors in all steps of testing. The barcoded labeling of samples help in better integration with the laboratory information system (LIS) and their easier identification. Automated pneumatic sample transportation system ensures faster delivery of samples to lab sections from the collection point. There is also an enhanced use of informatics continuum where there is a continuous recording of diagnostic test information.

The laboratory, thus, becomes an essential partner in clinical care. Automation adds value to the test result with faster TAT, improved accuracy, precision and safety. The electronic verification and authentication of results enhance the information value which ensures more accuracy and also better storage and faster retrieval of results.

Workflow Engineering

Diagnostic process is a series of discrete steps linked in a linear fashion but often separated physically and temporarily from each other. Automation can help in organising these various steps. Many laboratories still rely on paper documentation method to transmit sample information and test requisitions. This takes up significant amount of labour and time during data entry and also makes the process more prone to errors.

Manual sorting and processing of samples take up almost 25% of the total time for completing specimen analysis. An impediment to achieving maximum laboratory efficiency is the variability in specimen collection times, containers, labeling schemes, and transportation methods. The use of automation can simplify and organise workflow so that the entire process is more efficient. Automation is responsible for:

- Sample quality assessment at the beginning of the process
- Optimising specimen routing and scheduling
- Assessing process
- Specimen tracking
- Intelligent reporting
- Data retrieval and archiving
- Quality control documentation

Benefits of Automation

No laboratory and blood bank wants to go through the nightmarish experience of having delivered a wrong result to a patient because of which there had been devastating changes in treatment procedures. Most of these mistakes happen because of random human errors during registration, sorting and identification of samples, manual labeling, selection of test processes, and manual data entry from machines and delivery of test reports to patients.

This is where the automation comes in. The repetitive and monotonous nature of the work of technicians also makes them prone to committing mistakes, and more so when the sample load is more. Automation takes care of this. Another advantage is that it allows for a reduction in the manpower requirement and cost because of a leaner system.

CURRENT TECHNOLOGY AND THE FUTURE

Laboratory and blood bank automation, as mentioned before, is relatively new. With the advent of information technology (IT) and smarter machines, there is a corresponding increase in the demand for faster results by patients.

With capabilities of handling hundreds of samples in an hour, the current range of automated machines have eased the workflow, facilitated faster reporting, and automated the inventory and quality control management. Laboratory automation does not benefit the laboratory alone but benefits the patients as well by way of more accurate and faster reports. Chances of mistakes are greatly minimized.

All these help in delivering quality service to the patients and their treating physicians. The automation of laboratory testing does not mean there is no need for human intervention or expertise. Results, still need to be evaluated by medical technologists and other qualified laboratory professionals. What automation does is ease the concerns about error reduction, staffing, report timings, and safety.

Automation improves the objectivity and reproducibility of tests. It reduces human errors in patient identification and transcription errors. Documentation and traceability of tests, reagents and processes and archiving of results is another major advantage of automation. Shifting from manual methods to automation is a major undertaking for any transfusion service to provide quality patient care with lesser turnaround time for their ever increasing workload.

GR8LAB

Gr8Lab is a chemistry and turbidimetry analyser which accurately executes almost all-clinical chemistry and turbidimetry tests. Automatic selection of filters is all managed by the microprocessor. Gr8Lab is a fully open machine with 200 locations (Fig. 27.16).

Features

- 200 programming locations
- 30 µL flow cell
- 3000 tests internal memory
- Real-time graphs for two point and kinetic tests
- Auto diagnostics feature on start
- Programmable lamp save option
- Facility to print patients' details
- Dual measuring mode—flow cell and cuvette
- Acoustic signaling for ON and OFF of aspiration process
- Dedicated wash push button

Fig. 27.16: Gr8Lab

Technical Features

Light source	12V 20W halogen lamp with auto switch off facility for long time
Items	200 test parameters
Result storage	upto 3000 tests results
Optical system	340, 405, 492, 505, 546, 578, 630, 700 nm
Reaction volume	350 µL
Flow through cell	30 µL
Photometric range	0 – 2.5A
Software	Touch screen, english version
Display	Large TFT screen with touch screen
Printer	Built – in stylus/thermal printer
Serial output	RS-232 standard
Power supply	220V/110V ± 10% 50 Hz – 60 Hz
Dimension	350 mm (W) x 300 mm (D) × 180 mm (H)
Weight	5.8 kg

CORALAB TOUCH

Coralab touch is a semiautomatic analyzer with a 7-inch touchscreen display, 20 thermostat positions programmable from 25–37°C. It stores up to 180 test parameters and 6000 test results. It is a fully open system. Optics and automatic filter selection assures repeatable measurements. Results are displayed and printed out directly in the required measuring unit (Fig. 27.17).

Fig. 27.17: CoraLab touch

Technical Specifications

Opreating modes	Absorbance, End point, Fixed time, Kinetic, Multipoints, Sample blank
Items	180 test parameters
Result storage	up to 6000 test results
Reaction volume	400 µL
Flow cell	32 µL
Temperature control	37°C ± 0.2°C
Optical system	340, 405, 492, 510, 546, 578, 620 nm
Light source	6V, 10W Halogen lamp
Photomteric range	0 –3.0A
Software	Touch screen, english version
Display	7-inch touch screen
Printer	Built-in thermal printer
Serial output	RS – 232 standard
Power supply	220V/110V ± 10% 50Hz – 60Hz
Weight	6 kg

BT1500

The BT1500 is small in size, BIG in performance. BT1500 can run both clinical chemistry as well as turbidimetric tests. The BT1500 is equipped with a Bar-code reader for identification of samples and reagents and can be programmed to run up to 250 tests/hour in random, Batch as well as STAT test modes. With excellent on board washing system and on board cooling the BT1500 can accommodate 48 reagents and a total of 78 samples i.e 62 patient samples and 16 standards/controls and can be programmed with 3 level controls (3 known and 3 unknown). The excellent washing mechanism is designed to prevent carryover and maintains the 32 optical glass cuvettes in optimum state (Fig. 27.18).

Fig. 27.18: BT1500

Technical Specifications

Analyses type	Clinical chemistry, turbidimetry
Throughput	upto 250 Tests/hr
Water consumption	1125 mL/hr ± 20%
No. of reagents	48 total; 24 using 50 mL vessels and 24 using 10 or 20 mL vessels
Refrigerated reagent bay	Yes
Reaction volume	from 180 to 400 µL
Sample volume	from 1.8 to 100 µL
Reading cuvettes	32 non-disposable optical glass
Sampling arms	1 for serum and reagents
Sample tray # of positions	78 total; 62 for samples and 16 standards and controls
Test modes	Random access, batch, STAT
Test directory	Upto 500
Cuvette temp. control	Peltier System OFF/30/32/37°C (±0.2°C)
Vacuum pump system	Internal
Photometer	Solid state 10 channel + reference channel (340, 380, 405, 436, 480, 510, 546, 578, 630, 700 nm)
Photometer linearity	up to 3.0 OD
Photometer sensitivity	±0.001 Abs
Diluter	1 – long life ceramic 340 µL
Diluter precision	±0.1% fs
Diluter resolution	0.1 µL
Bar code	Optional, both for reagents and samples
Built in QC program	3 levels known, 3 levels unknown
Maintenance	Software assisted
Computer	Optional external PC
PC interface	USB 2.0
Power supply	Universal input 100/240 Volt, 50–60 Hz, 300 W
Dimensions	Height – 20″, Width – 30.7″, Depth – 22.8″ (51 × 78 × 58 cm)
Weight	53 kg

COUNCELL PENTA 2.0

Councell Penta 2.0, 5 Diff auto hematology analyzer is specially designed in accordance to international standards. The system has state of art technology, customized user friendly software and robust hardware. The system is based on principle of triangle laser scatter

Flow cytometry for WBC and its differential count, electrical impedance for RBC andPLT and cyanide free colorimetric method for HGB estimation. The system offers unique QC and calibration programs. It uses only 3 reagents for analysis of sample and offers a throughput of 60 sample/hour. The instrument possesses intelligent sleep mode, smart prompt and one touch error resolving capability. The system has 10.4 inches colour touch screen and in built thermal printer (Fig. 27.19).

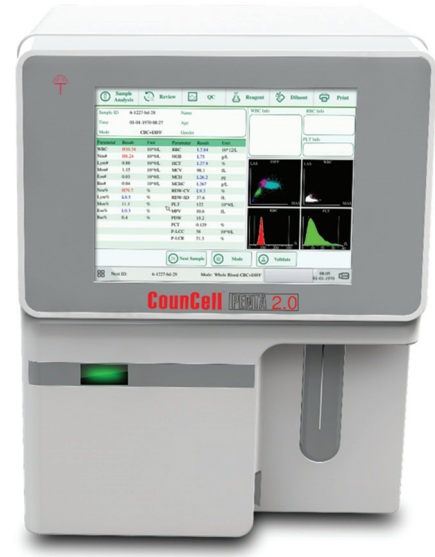


Fig. 27.19: CounCell Penta 2.0

Technical Specification

Principle	Flow Cytometry + Tri angle laser scatter method for WBC and its differential counting. Electrical impedance method for RBC and PLT counting and cyanide free colorimetric method for HGB
Reportable parameters	29 parameters, consisting of 4 RUO parameters
	WBC, LYM#, LYM%, MON#, MON%, NEU#, NEU%, EOS#, EOS%, BAS#, BAS%, RBC, HGB, HCT, MCV, MCH, MCHC, RDW-SD, PLT, MPV, PDW, PCT, PLCC, PLCR, LIC%, ALY# & ALY%.
	4 Scattergrams, 2 Histograms for RBC and PLT
	Alarm for abnormal erythrocytes, leukocytes and platelet.
Throughput	Up to 60 samples per hour
Reagent	3 Routine reagents: Diluent, LH lyse and Diff Lyse
Data storage capacity	60,000 test values with parameters, histograms, scattergrams and patient information.
Sample volume	20 µL
Quality control	2 QC method; L-J and X-B QC with appropriate QC charts
Calibration	3 types of calibration: Manual, auto calibration with calibrators and auto calibration using fresh samples
Power requirement	100V-240 VAC; 50/60 HZ
Operating environment	Temperature: 10–30°C
	Relative humidity: 30 – 85%
	Atmospheric pressure: 70–106 kPa
Dimension	430 mm (L) × 350 mm (W) × 450 mm (H)
Weight	28 kg

COUNCELL 23 EXCEL

CounCell 23 Excel 3-Diff Auto Hematology Analyzer is specially designed in accordance to international standards. The system has state of art technology, customised user friendly software and robust hardware. The system is based on principle of electrical impedance for counting of WBC, RBC and PLT and cyanide free colorimetric method for HGB estimation. The system comes with special counting mode for precise estimation of clinically important WBC and PLT. The system offers unique QC and calibration programs. It uses only 2 reagents for analysis of sample and offers a through put of 60 samples/hour. The instrument possesses intelligent sleep mode, smart prompt and one touch error resolving capability. The system has 10.4 inches' colour touch screen and in built thermal printer (Fig. 27.20).

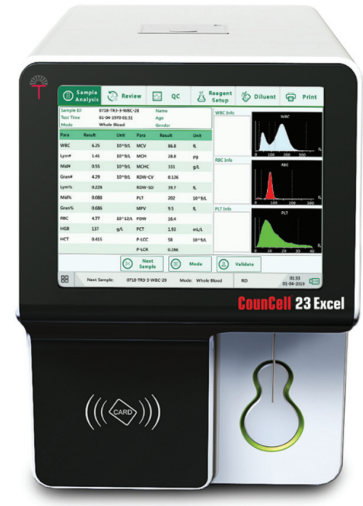


Fig. 27.20: CounCell 23 excel-3

Technical Specification

Principle	Electrical impedance method for WBC/RBC/PLT
	Cyanide free colorimetric method for HGB
Reportable parameters	21 parameters consisting: WBC, LYM%, MID%, GRAN%, LYM#,
	MID#, GRAN#, RBC, HGB, HCT, MCV, MCH, MCHC, RDW-CV, RDW-SD, PLT, MPV, PDW, PCT, P-LCR, P-LCC.
	3-Histograms for WBC, RBC and PLT
	Alarm for abnormal erythrocytes, leukocytes and platelet.
Throughput	60 samples per hour
Reagent	2 routine reagents: Diluent and lyse
Sample volume	9 µL
Data storage capacity	Internal memory can store 60,000 test values with parameters, histograms and patient information
Quality control	2 QC method; L-J and X-B QC with appropriate QC charts
Calibration	3 types of calibration: Manual, autocalibration using calibrators and auto-calibration using fresh samples.
Power requirement	100v–240VAC; 50/60Hz
Operating environment	Temperature: 10–30°C
	Relative humidity: 30–85%
	Atmospheric pressure: 70–106 kPa
Dimension	430 mm (L) × 280 mm (W) × 400 mm (H)
Weight	17.5 kg

CORALYZER SMART

Coralyzer Smart is a fully automatic, random access analyzer with a throughput of 200 tests/hour. It can run both clinical chemistry and turbidimetric tests. It can accommodate 80 reagent positions and 40 sample positions. The instrument comes with teflon coated

mixer which prevents water dropping and efficient cleaning with dedicated maintenance solutions to reduce carryover and improve life of cuvettes (Fig. 27.21).

Fig. 27.21: Coralyzer Smart

Technical Specifications

Machine type	Random access analyzer
Test speed	200 tests/hour
Test principle	Colorimetric, turbidimetric
Test method	End point, fixed time, kinetic, two point
Sample tray	40 samples position
Sample cup type	Micro cup
Sample volume	2–30 µL, step by 0.1 µL
Sample probe	Liquid surface detection
Reagent tray	80 reagent positions (40 for R1 and 40 for R2)
Reagent volume	20–300 µL, step by 1 µL
Reagent needle	Liquid surface detection
Reaction tray	44 reaction cuvettes
Reaction volume	150–330 µL
Temperature	Peltier pad incubation system
Mixer	Paddle type mixing
Light source	Halogen lamp
Wavelength	340–700 nm
Absorbance range	0-4.0 Abs
Operating system	Windows XP, Windows 7, Windows 8, Windows 10
Power supply	AC 110/220V ± 10%, 50/60 Hz, 650 w
Dimension	L = 75 cm × W = 56 cm × H = 54 cm
Weight	48.4 kg

CORALYZER JUNIOR

Coralyzer junior is a fully automatic, random access analyzer with a throughput of 100 tests/hour. It can run both clinical chemistry and turbidimetric tests. It can accommodate 80 reagent positions and 40 sample positions. The instrument comes with teflon coated mixer which prevents water dropping (Fig. 27.22).

Technical Specifications

Machine type	Random access analyzer
Test speed	100 tests/hour
Test principle	Colorimetric, turbidimetric
Test method	End point, Fixed time, Kinetic, Two point
Sample tray	40 samples position
Sample cup type	Micro cup
Sample volume	2–30 µL, step by 0.1 µL
Sample probe	Liquid surface detection
Reagent tray	80 reagent positions (40 for R1 and 40 for R2)
Reagent volume	20-300µl, step by 1 µL
Reagent needle	Liquid surface detection
Reaction tray	50 reaction cuvettes (removable)
Reaction volume	150–330 µL
Temperature	Peltier pad incubation system
Mixer	Paddle type mixing
Light source	Halogen lamp
Wavelength	340–700 nm
Absorbance range	0–4.0 Abs
Operating system	Windows XP, Windows 7, Windows 8, Windows 10
Power supply	AC 110/220V ± 10%, 50/60 Hz, 650 w
Dimension	L = 75 cm × W = 56 cm × H = 54 cm
Weight	48.4 kg

Fig. 27.22: Coralyzer Junior

TURBODYNE SC

Turbidimetry is the most convenient method of quantitating immunoassay parameters like HbA1c, CRP, D dimer, ferritin, etc. on chemistry analysers. However, when using conventional turbidimetry, it is important to follow the correct programming protocols to program the reagents on analysers. Secondly it is also important to follow the correct reagent sample ratios for maintaining the performance characteristics like sensitivity, specificity and linearity of the reagents. Thirdly, it is very important to mix sample and reagents and monitor the delay and reaction time for measuring reactions accurately. Person to person variations in these variables can lead to calibration errors, test to test variations. However, with the advent of smart card technology all these variables are brought under control.

Turbodyne SC is a smart card technology based immunoturbidimetry turbidimetry analyzer. It is a secure way of handling immunoturbidimetry reagents. It is a system which utilizes smart card (RFID) and therefore does not require any programming and calibration to run assays on it. Turbosmart is compatible with Turbosmart reagents which are supplied with smart card encrypted with lot specific calibration and programming data for the respective reagents. The system brings under control manual errors associated with mixing, calibration and reading usually encountered with conventional turbidimetry reagents run on a biochemistry analyser through preprogrammed mixing and reading times (Fig. 27.23).

One smart card is provided with each reagent pack. The laboratarian does not have to prepare the calibration curve, or program the assay. The smart card contains the calibration and the applications to perform the test. The use of smart card based testing eliminates person to person variations caused during aspiration, mixing and reading time. These parameters are now completely under instrument control thereby improving precision.

SC Tecnnology Benefits

- Optical system: Detector at 180° SECURES turbidimetric measurements
- Lot specific assay smart card SECURES master calibration, optimised mixing and accurate reading of reactions.
- Touch screen display SECURES user friendly operations with online reaction curve

Fig. 27.23: Turbodyne SC

- QC software with levey jennings SECURES reliable reporting
- Electronic pipette SECURES auto start of testing time
- Optimix mixer SECURES automatic onboard mixing of reaction mixture
- Curve correction SECURES sample correlation
- Unique cuvette design SECURES optimized onboard mixing

Disposable cuvettes SECURES no carryover effects, reduces labor and minimizes biohazard.

HEMOSTAR XF

Hemostasis assessment is critical for diagnosis of thrombosis, Bleeding or monitoring anticoagulation therapy. Measuring blood clotting factors prior to surgery is a preventive step to avoid the possibility of excessive bleeding. Secondly, in lieu of changing lifestyle, the incidence of thrombotic conditions are increasing leading to increase in the usage of anticoagulants. The prothrombin time (PT) is the most widely used test followed by the partial thrombin time test (APTT) for estimating the factors of extrinsic and intrinsic pathways. The PT test is the most widely used test for monitoring oral anticoagulant therapy and the APTT for heparin therapy.

Manual method of testing coagulation parameters requires good skill to manage the incubation time, temperature, mixing of reagents and samples for obtaining correct clotting times. Testing on coagulation analysers will help bring under control the incubation time, temperature and mixing of reagent and samples leading to accurate clotting times even when operated by staff with average skills.

The coagulation analysers currently available in the market can be divided into analyzers using to different principles of clot detection:

a. Turbodensitometric or optomechanical method of measurement (where mixing takes place during the running of reaction for sensitive clot detection.
b. Optical method of measurement where mixing does not take place during the running of reaction.

It must also be noted that the sensitivity of the PT reagent is influenced with the coagulation analyser used and therefore it is ideal to use instruments that are synergized with the reagents to obtain correct INR when performing PT test

The optomechanical method of measurement is more preferred as it is less affected by icteric and lipemic samples compared to optical systems.

Hemostar XF 1.0

It is an indigenized single channel coagulation analyser, based on time-tested opto-mechanical (turbodensitometric) measuring principle, suitable for sensitive clot detection. The instrument is suitable for measuring clot based assays such as PT, APTT, TT, Fibrinogen, factor and other clot based assays. The instrument is in synergy with India's most widely used liquid stable PT reagents UNIPLASTIN and LIQUIPLASTIN known to foster safe oral anticoagulation in every nook and corner of our country for over 2 decades.

Hemostar XF 1.0 is provided with 18 incubation positions at 37° ± 1°C (16 specimen position and 2 reagent positions).

It is the only semi-automated coagulation analyzer available with 50 programmable locations. It has calibration facility for 2–7 point calibration with plot. Hemostar XF 1.0 an inbuilt thermal printer and RS232 port for data transfer.

Hemostar XF 2.0

Hemostar XF 2.0 is an indigenized two channel coagulation analyser, based on time-tested opto-mechanical (turbodensitometric) measuring principle, suitable for sensitive clot detection. Hemostar XF 2.0 is capable of measuring 2 different assays simultaneously. The instrument is suitable for measuring clot based assays such as PT, APTT, TT, Fibrinogen, factor and other clot based assays. The instrument in synergy with the India's most widely used liquid stable PT reagents UNIPLASTIN and LIQUIPLASTIN known to foster safe oral anticoagulation in every nook and corner of our country for over 2 decades.

Hemostar XF 2.0 is provided with 18 incubation positions at 37° ± 1°C (16 specimen position and 2 reagent positions).

It is the only semiautomated coagulation analyser available with 50 programmable locations. It has calibration facility for 2–7 point calibration with plot and 3 level QC with Levey Jennings plot/Hemostar XF 2.0 an inbuilt thermal printer and RS232 port for data transfer.

Hemostar 4CA

Automation is very important to increase productivity, reduce human errors associated with reagent/sample pipetting, incubation time, etc. Automation also reduces repeated contact with specimens. There are certain primary features in an automation which improve the accuracy of test results like pipetting, incubation time, on-board cooling) and secondary features which do not influence the test results but improve throughput, convenience and safety. As the secondary features (no of sample/reagent positions, through put, cap piercing) increase the cost of the instrument also increases. Currently more than 90% of laboratories are not able to fulfill their dream of automation in coagulation mainly due to high instrumentation cost and low shelf life of reagents making return on investment difficult.

Considering the fact that mostly PT, APTT and fibrinogen are the most widely used parameters automating these would serve the purpose of most laboratories.

Hemostar 4CA is an ideal instrument which fulfills the primary features (features which improve the accuracy of test results) of a coagulation automation thereby making it cost effective solution. It can automate the basic coagulation tests of primary coagulation cascade, i.e prothrombin time, partial thromboplastin time and fibrinogen (extrinsic, intrinsic and the common pathways). Hemostar 4 CA is a suitable for automating the basic coagulation parameters (PT, APTT and fibrinogen) which constitutes 95% workload of a routine laboratory (Fig. 27.24).

Hemostar 4 CA has the following primary features:

- 4 measuring channels with photo-optical measuring principle
- Test menu: PT, APTT and FIB
- Automatic/manual calibration function
- Continuous sample loading
- Auto sample dilution and rerun
- On-board reagent cooling

Fig. 27.24: Hemostar 4CA

- Tilt reagent position for maximum reagent utilization
- Probe with liquid level detection and 37°C incubation for reagent
- Wash station for internal and external washing of probes
- Multilevel QC with LJ graph
- Manual testing mode for back-up
- User friendly windows based software

Euphoreia 4.1

Four plate ELISA processor

- Fully automated high speed robotic modular ELISA processor
- Fast and accurate 2 channel pipetting system
- Intelligent microplate transporter enhances the speed of the processor
- 4 Plate positions with independent incubator and shaker
- 8 Channel reading module provides fast and accurate results
- Nonconductive tip ensure zero carry over
- Powerful windows based operating system

Matrix™ Gel System

- Indigenously developed technology
- High sensitivity and specificity
- Easy and convenient to perform
- Reproducible results
- CE marked products

Gel Card Centrifuge

Table top model

- Back-illuminated LCD (2 lines 16 characters)
- Microprocessor based controller with programmable operation
- Acrylic see-through cover with electronic door locking with open lid detection
- Lid lock on display (If door is not closed properly)

Hand-held Vein Finding Device

Veinspy™

Hand-held vein finding device (Fig. 27.25).

- Orange light for viewing superficial veins
- Red light for viewing deeper veins
- Mains on and low battery indicator
- User-friendly operation
- Battery back up for 90 minutes
- Disposable plastic sleeve to prevent patient to patient contamination

Fig. 27.25: VeeinSpy

Application of VeinSpy™

- Easy visualization of veins in adult pediatric and geriatric patients with difficult venous access
- Clear visualization of veins for obese and dark skin patient
- Clear visualization of veins before starting of IV therapy

Index

Chapter 1: History and Scope 1

Chapter 2: Blood Formation 4

Basophils and mast cells 24
Development of blood cells 6
  adhesion molecules 6
  hematopoietic stem and progenitor cells 6
  in an adult 6
Eosinophils 24
Erythropoiesis 9
  erythroid precursors 12
  erythron concept 9
  erythron: proliferation and maturation 11
  maturation chart 9
Leukopoiesis 16
Lymphocytic series 25
  activated and reactive lymphocytes 26
  large lymphocyte 25
  lymphoblast 25
  plasmablasts and proplasmocytes 27
  plasmocytes 27
  prolymphocyte 25
  small lymphocyte 26
Lymphokines and monokines 30
  B cells 31
  interleukin-1 (IL-1) 30
  interleukin-2 (IL-2) 30
  interleukin-4 (IL-4) 30
  interleukin-6 30
  interleukins 7, 9–13 30
  lymphocyte circulation 32
  lymphocyte proliferation and differentiation 31, 32
  lymphocytes 30
  natural killer (NK) cells 31
  T cells 31
Macrophages 29
  control of leukopoiesis 30
Monocytic series 28
  lymphocytic, monocytic and plasma cell series 29
  monoblast 28
  monocyte 28
  promonocyte 28
Myelopoiesis 16
  development of a mature neutrophil 16
  development of neutrophils and other cells 17
  myeloid series 16
Myeloid series 17
  band neutrophils 23
  metamyelocyte 20
  myeloblast 17
  myelocyte 19
  neutrophil granule formation 20
  polymorphonuclear neutrophils 23
  promyelocytes 19
Platelet series 33
  control of platelet formation 35
  mature platelet 35
  megakaryoblast 33
  megakaryocyte 33
  promegakaryocyte 33
Sites of blood formation 4
  abnormal sites 5
    examples 6
  distribution of active bone marrow in an adult 5
  normal sites 5
Stages of normoblastic differentiation 12
  action of EPO 14
  control of erythropoiesis 14
  early normoblast (basophilic normoblast or basophilic erythroblast) 13
  erythropoietin 14
  ineffective erythropoiesis 16
  intermediate normoblast (polychromatophilic normoblast or erythroblast) 13
  late normoblast (orthochromatic normoblast or erythroblast) 13
  pronormoblast 12
  regulation of erythropoiesis 14
  reticulocyte 13
  substances needed for erythropoiesis 15

Chapter 3: Red Blood Cells 36

Anemia 54
  cardiorespiratory system 58
  cardiovascular adjustments 56
  classification of anemia based on pathogenesis and etiology 62
  classification 62
  clinical features 57
  compensatory physiological adjustments 55
  definition 54
  gastrointestinal system 60
  genitourinary system 60
  history and physical examination 60
  kinetic classification of anemia 64
  kinetic classification 63
  morphological classification 63
  neuromuscular system 60
  other signs 60
  pathophysiology of anemia 55
  retinopathy 60
  skin 59
  symptoms and signs 57
Hemoglobin function 43
  active red cell metabolic pathways 44
  deoxyhemoglobin 43
  intravascular hemolysis 49
  normal extravascular hemolysis 48
  normal hemoglobin–oxygen dissociation curve 43
  nutritional and other requirements of a red cell 49
  oxyhemoglobin 43
  RBC senescence/aging 47
  reactions within RBC to prevent accumulation of oxidants 46
  red cell metabolism 46
  right-shifted hemoglobin–oxygen dissociation curve 44
Hemoglobin structure and function 41
  globin synthesis 42
  hemoglobin synthesis 41
  iron delivery and supply 41
  synthesis of hemoglobin in a reticulocyte 41
  synthesis of protoporphyrins 41
Normal red cell values 49
RBC count 50
  diurnal and day-to-day variation 53
  hematocrit (packed cell volume) (PCV) 51

hemoglobin 50
mean cell (corpuscular) volume (MCV) 51
mean cell diameter (erythrocyte: dry film) 53
mean cell hemoglobin (MCH) 52
mean cell hemoglobin (MCH) 53
mean cell hemoglobin concentration (MCHC) 52
mean cell hemoglobin concentration (MCHC) 53
mean cell volume (MCV) 53
red cell absolute values/ indices 53
reticulocyte count blood 53
Red blood cells 36
deformability 40
historical aspects 36
integral membrane proteins 39
lipids 38
peripheral proteins 40
permeability 40
physiology of the red blood cells 37
RBC membrane proteins 39
red cell membrane 37
shape 36
structure 36

Chapter 4: Basic Principles in Diagnosing and Treating Anemias 65

Cause of anemia 67
Examination of blood film 68
Is the patient anemic 65
Treatment 71
hematinics 72
Type of anemia 66

Chapter 5: Iron Deficiency Hypochromic Anemia 73

Absorption of iron 74
Bone marrow iron 95
criteria for grading iron stains in bone marrow aspirated 95
Hemosiderosis 90
Hemochromatosis 92
Hemoglobin synthesis in the developing red cell 73
Investigations of a patient 90
Iron deficiency anemia 81
bone marrow 86
clinical features 83
clinical presentation 83
etiology of iron deficiency anemia 81
management 87
peripheral smear 86
serum biochemistry 86
treatment 86
Iron metabolism 73
Iron overload 90
Iron balance 76
iron requirements in males and females of various ages 76
Mechanism and control of iron absorption 74
iron absorption, its control and utilization 75
iron absorption and losses 75
Pathogenesis of iron deficiency anemia 76
alveolar hemorrhage 79
blood donation 79
blood loss in infancy 81
blood loss 78
chronic renal failure treated with hemodialysis 79
decreased total body iron at birth 80
diet in infancy and childhood 81
fetomaternal transfusion 80
gastrointestinal bleeding 78
hemoglobinuria 79
hemostasis disorders 79
hereditary hemorrhagic telangiectasia 79
impaired absorption 77
infancy 79
iron deficiency anemia 77
iron deficiency: stages of development 76
latent iron deficiency 77
menstruation 78
nosocomial blood loss 79
pregnancy and lactation 81
prelatent iron deficiency 77
prevalence 77
Plummer–Vinson syndrome (Paterson-Kelly syndrome) 90
Radioactive ^{59}Fe studies 94
Relevant normal values 73
Sideroblastic anemias 92
acquired primary idiopathic sideroblastic anemia 94
classification 93
hereditary sideroblastic anemia 94
pseudothalassemia 94
secondary sideroblastic anemia 94
treatment 94
types 93

Chapter 6: Megaloblastic, Macrocytic Anemias 96

Abnormal vitamin B_{12} transport 120
Bone marrow 117
dyserythropoiesis 117
megakaryocytes 117
WBC series 117
Causes of macrocytosis other than megaloblastic anemia 122
Clinical manifestations 99
Course, prognosis, and complications 103
Diagnosis of pernicious anemia 118
Drug-induced disorders of dna synthesis 121
Erythrocyte kinetics, iron and bilirubin metabolism 101
epithelial changes 102
gastrointestinal secretion 102
serum LDH and other enzymes 102
Etiology 106
vitamin B_{12} balance and deficiency 106
Etiology and pathogenesis 98
genetic factors 98
gastritis 98
immunologic mechanism 99
Folate antagonists 121
Folate deficiency 110
alcoholism and cirrhosis 110
causes of folate deficiency 110
causes of folate deficiency 113
clinical manifestations 114
congenital folate malabsorption 111
dietary deficiency 110
drug-induced folate deficiency 111
FIGLU test 115
folic acid balance and deficiency 113
gluten-induced enteropathy 112
infants and children 111
management 115
pregnancy 110
radioactive folic acid test 115
rapid cellular proliferation 111
serum folate assay 114
tests for diagnosing folate deficiency 114
tropical sprue and gluten-sensitive enteropathy 112
tropical sprue 112
Folate kinetics 98
Homocystinuria and methylmalonyl aciduria 119
Inherited disorders altering DNA synthesis 119

Management 108
response to therapy 109
treatment (basic principles) 109
Megaloblastic, macrocytic anemias 96
relevant normal values 96
Mechanism of anemia in megaloblastic anemia 117
biochemical basis of megaloblastic anemia 118
dU suppression test 118
vitamin B_{12} deficiency impairs folate availability 118
Nitrous oxide 122
Other biochemical findings 103
Other conditions associated with vitamin B_{12} deficiency 103
chronic pancreatic disease 105
dietary deficiency of vitamin B_{12} 104
diseases affecting the ileum 104
drug-induced vitamin B12 malabsorption 105
familial selective vitamin B12 malabsorption 105
fish tapeworm disease 104
functionally abnormal intrinsic factor 105
gastric surgery 103
hemodialysis 105
HIV infection 105
intestinal disorders 104
small bowel bacterial overgrowth 104
tropical sprue and celiac disease 105
Zollinger–Ellison syndrome 104
Other drugs and toxins 122
Orotic aciduria 120
Peripheral blood findings in vitamin B_{12} or folic acid deficiency 115
Recapitulation 122
Schilling test 107
variant 108
Signs and symptoms of pernicious anemia 118
Tests for diagnosing vitamin B_{12} deficiency 107
radioactive vitamin B_{12} absorption test 107
Thiamine responsive megaloblastic anemia 121
Vitamin B_{12} kinetics 96
Vitamin B_{12} deficiency 98

Chapter 7: Symptomatic Anemias 129

Acute hemorrhagic anemia 129
Addison's disease 147
Anemia associated with endocrine disorders 145
Anemia associated with secondary carcinoma of the bone marrow 140
Anemia of chronic disorders 150
Anemia of infection 132
Blood picture 129
Chronic renal failure 134
Clinical aspects 130
Clinical description 130
Collagen disease 143
Endocrine disorders 145
Erythropoietin resistance 138
peritoneal dialysis 138
Etiological classification of normocytic anemia 155
algorithm for investigation of a patient with normocytic, normochromic anemia 156
Hematologic findings 129
Hyperparathyroidism 147
Hyperthyroidism 146
Hypogonadism 146
Hypopituitarism 147
Investigations of a patient with normocytic anemia 157
Liver disease 141
Malignancy 138
Management 131
Mechanisms of anemia in malignancy 139
Myxedema 145
Normocytic and normochromic anemias 155
Nutritional anemia of pregnancy 149
Pathophysiology 131
Physiological anemia of pregnancy 148
Protein malnutrition 148
Scurvy 148
Systemic lupus erythematosus 144
LE cell phenomenon 144
Treatment 132
Tuberculosis 133

Chapter 8: Hemolytic Anemias 158

Abnormalities of purine and pyrimidine nucleotide metabolism 217
adenylate kinase deficiency 219
Acanthocytic disorders 199
abetalipoproteinemia 199
echinocytic disorders 201
familial lecithin-cholesterol acyl-transferase (LCAT) deficiency 201
MacLeod phenotype 200
Acquired hemolytic anemia 165
Agents that may cause hemolytic anemia (drugs and chemicals) 236
Approach to diagnosis 176
Autoimmune hemolytic anemia (AIHA) 219
Cardiac hemolytic anemia 243
Classification 160
extracorpuscular 160
intracorpuscular 160
Classification 219
Conditions that can sometimes be mistaken for hemolytic anemia 176
Cold antibody AIHA 227
cold hemagglutinin disease (CHAD) 227
paroxysmal cold hemoglobinuria (PCH) 231
paroxysmal nocturnal hemoglobinuria (PNH) (marchiafava-micheli syndrome) 233
Chronic congenital hemolytic anemia 163
cholelithiasis 165
crises 163
degree of anemia 163
jaundice 163
leg ulcers 165
skeletal abnormalities 165
splenomegaly 164
Clinical manifestations 163
Clostridium welchii infection 246
Coagulation 245
Definition 158
associated with certain infections 159
caused by certain chemical agents 159
compensated hemolytic anemia 159
hemolytic anemias characterized 159
paroxysmal nocturnal hemoglobinuria 159
thermal injury 159
Disseminated carcinoma 245
Drug-induced autoimmune hemolytic anemia 241
Drug-induced hemolytic anemia due to hereditary red cell enzyme deficiencies 239
Drug-induced hemolytic anemia due to unstable hemoglobins 239
Drug-induced immune hemolytic anemia 240
Etiopathogenetic classification of hemolytic disorders 161

abnormalities of erythocyte nucleotide metabolism 161
acquired hemolytic anemias 162
chemicals, drugs and venoms 162
defects in erythrocyte membrane 161
defects in globin structure and synthesis 161
deficiencies of enzymes involved in the pentose phosphate pathway and in glutathione metabolism 161
deficiency of erythrocyte glycolytic enzymes 161
immunohemolytic anemia 162
infectious agents 162
traumatic and microangiopathic hemolytic anemias 162

Ferrokinetic studies 172

Glucose-6 phosphate dehydrogenase deficiency and related disorders of hexose monophosphate shunt and glutathione metabolism 202
Glucose-6-phosphate dehydrogenase deficiency 202
favism 208
G6PD enzyme and its variants 204
neonatal hyperbilirubinemia 208

Hemolytic anemia associated with bacterial infections and parasitic infestations 246
Hemolytic anemia due to direct toxic action 238
Hemolytic anemia due to drugs and chemicals 236
Hemolytic anemias caused by drugs 236
Hemolytic uremic syndrome 244
Hereditary elliptocytosis (HE) syndromes 191
clinical and laboratory findings of HE syndromes 193
common HE 193
elliptocyte formation due to membrane abnormalities 193
glycophorin C deficiency 193
HE variants—prevalence 191
Hereditary hemolytic anemias associated with abnormalities of erythrocyte glycolysis and nucleotide metabolism 212
Hereditary pyropoikilocytosis (HPP) 194
homozygous (doubly heterozygous) HE 194
membrane protein defects 192
pathogenesis 192
protein 4.1 defects 192
southeast asian ovalocytosis (SAO) 195
spectrin abnormalities 192
spherocytic elliptocytosis (SE) 195
Hereditary spherocytosis (HS) 184
diagnosis 189
erythrocyte abnormalities 186
laboratory findings 188
pathogenesis 185
role of the spleen in the pathophysiology 186
treatment 190
Hereditary spherocytosis and other anemias due to abnormalities of the erythrocyte membrane 183

Immunological drug-induced hemolytic anemia 239

Laboratory manifestations 166
bone marrow 172
catabolism of heme 167
chemical markers of cell age 171
conditions associated with unconjugated hyperbilirubinemia 167
disappearance of haptoglobin 168
erythrocyte survival 166
glycosylated hemoglobin (Hb A1) 168
hemoglobinemia 169
hemoglobinuria 169
laboratory signs of accelerated erythropoiesis 170
laboratory signs of accelerated red cell destruction 166
lactate dehydrogenase (LDH) 168
methemalbumin and hemopexin 170
other morphologic findings in the blood 171
rate of heme catabolism 167
serum bilirubin 167
signs of accelerated erythropoiesis 170
signs of excessive red cell destruction 166
signs of intravascular hemolysis 169
urine iron excretion 169
Laboratory tests 172
acanthocytes 173
autoagglutination 174
autohemolysis 175
Coombs' test (antiglobulin test) 174
echinocytes 173
elliptocytes 173
osmotic fragility test 174
schistocytes 173
sickle cells 173
spherocytes 172
stomatocytes 173
target cells 173
tests for hemolytic disorders associated with heinz body formation 175
Lead poisoning 247

Malaria 246
Malignant hypertension and eclampsia 245
March (exertional) hemoglobinuria 245
Mechanical hemolytic anemia 242
Microangiopathic hemolytic anemia 243

Pyruvate kinase deficiency 214

Recapitulation in brief 179
Red cell membrane: general considerations 183
Related disorders of HMP shunt and glutathione metabolism 210
6-Phosphogluconate dehydrogenase deficiency 210
defects in glutathione synthesis 211
glutathione peroxidase deficiency 210
glutathione reductase deficiency 210
glutathione S-transferase deficiency 212
glutathione synthetase deficiency 211
γ-glutamyl-cysteine sysnthetase (g-GCSynth) deficiency 211

Stomatocytic disorders 196
hereditary stomatocytosis 197
hereditary xerocytosis 198
intermediate stomatocytic syndromes 198
Rh null disease 198
Summary of clinical investigations 248

Thrombotic thrombocytopenic purpura and disseminated intravascular

Warm antibody AIHA 220

Chapter 9: Disorders of Hemoglobin Structure and Synthesis 250

Abnormal hemoglobins and the hemoglobinopathies 252
Alpha-thalassemia and other hemoglobinopathies 282
Alpha-thalassemia trait 280
Classification of thalassemias 269
Classification 268
  clinical 269
  α-thalassemia 270
  β-thalassemia 269
Combination of HBS with other hemoglobinopathies 262
Delta-beta thalassemia 278
Disorders of hemoglobin structure and synthesis 250
Genetic regulation 251
Hemoglobin C hemoglobinopathies 263
Hemoglobin D hemoglobinopathies 264
Hemoglobin E hemoglobinopathies 264
Hemoglobin H disease 281
Hemoglobin structure 251
Hemoglobin types 250
Hemoglobin-barts hydrops fetalis 282
Hemoglobinopathies associated with polycythemia 266
Hereditary methemoglobinemia 283
Hereditary persistence of fetal hemoglobin (HPFH) 279
Laboratory diagnosis 253, 265, 267, 268
  alkali denaturation 255
  clinical and laboratory findings 267
  demonstration of HbH inclusions 268
  Heat instability test 265
  Heinz bodies 265
  hemoglobin electrophoresis 254, 255
  hemoglobin electrophoresis 267
  investigations depending on physicochemical properties of abnormal hemoglobins 254
  isopropanol precipitation test 265
  oxygen dissociation studies 267
  routine hematological and biochemical tests 254
  routine hematological tests 268
  the acid elution test 255
Methemoglobinemia 282
Normal hemoglobin 250
Sickle cell anemia 259
  abdominal crises 260
  aplastic crises 260
  blood picture 261
  bone and joint crises 260
  CNS crises 260
  hand and foot syndrome 259
  hematologic crises 260
  hemolytic crises (hyperhemolytic crises) 260
  infectious crises 261
  megaloblastic crises 260
  pulmonary crises 260
  sickle cell crises 259
  splenic sequestration crises 260
  treatment 262
  vaso-occlusive crises 259
Sickle cell HbC disease 263
Sickle cell HbD disease 263
Sickle cell trait (AS) 258
Sickle cell β-thalassemia 262
Sickle hemoglobinopathies 255
Sulfhemoglobinemia 284
Thalassemias (reduced synthesis rate) 267
The hemoglobin-Lepore syndromes 278
Toxic methemoglobinemia 282
Types of structural abnormalities 253
Unstable hemoglobin hemoglobinopathies 265
Uptodate advances in hemoglobinopathy screening and diagnosis 284
  alkaline globin chain electrophoresis 287
  allelic discrimination using real-time PCR 289
  capillary zone electrophoresis 287
  cellulose acetate electrophoresis (alkaline) 286
  citrate agar electrophoresis (acid) 287
  considerations for all polymerase chain reaction (PCR)-based assays 289
  DNA sequencing 290
  high performance liquid chromatography (HPLC) 285
  isoelectric focusing (IEF) 285
  molecular methods 288
  restriction fragment length polymorphism (RFLP) 289
β-thalassemias 270
β-thalassemia minor (TRAIT) 271
β-thalassemia major 272
  measurement of globin chain synthesis 277
  analysis of fetal DNA 277
α-thalassemia 279
  classification 279

Chapter 10: Aplastic Anemia, Pancytopenia 293

Acquired pure red cell aplasia 308
Antithymocyte globulin administration 307
  anabolic agent administration 307
  corticosteroid administration 307
Aplastic anemia and infectious hepatitis 300
Aplastic anemia 297
Bone marrow infiltration or replacement 310
Chemical exposure 300
Classification 297
Diagnosis of pancytopenia 294
Differential diagnosis of pancytopenia 308
Drug idiosyncrasy 298
Etiology 294
Etiology of aplastic anemia 298
Fanconi's anemia 301
Investigation of cases with pancytopenia 294
  blood examination 295
Measures to accelerative recovery from pancytopenia 306
  bone marrow transplantation 306
Multiple myeloma 310
Myelodysplastic disorders 310
Pancytopenia 293
Paroxysmal nocturnal hemoglobinuria 311
Pure red cell aplasia 308
  congenital or diamond–blackfan anemia 308
Systemic lupus erythematosus 311

Chapter 11: White Blood Cells 312

Bone marrow plasmacytosis 322
Chronic idiopathic neutropenia (agranulocytosis) 327
Disorders of phagocytic leukocytes characterized by morphologic changes 330
  Alder–Reilly anomaly 331
  Chediak–Steinbrinck–Higashi anomaly 332

lymphocyte vacuolation 332
May–Hegglin anomaly 331
mucopolysaccharidoses VI and VII 332
Pelger–Huet anomaly 331
pseudo or acquired Pelger–Huet anomaly 331
Drug-induced neutropenia 326
Felty's syndrome 328
Function of white blood cells 315
Infectious mononucleosis 329
Paul–Bunnell test 330
Leukocyte surface antigens 316
Leukoerythroblastic change 323
Metabolic and enzymatic characteristics of white blood cells 313
Neutropenia and agranulocytosis 324
Neutrophil alkaline phosphatase 323
Normal white cell values 316
Pathological variations in white cell counts 317
basophilia 321
basophilopenia 321
eosinopenia 322
eosinophilia 319
lymphocytosis 320
lymphopenia 322
monocytosis 320
neutrophilia 317
Physiology of white blood cells 312
Production and life span of white blood cells 312
Sites of production 312

Chapter 12: Leukemias 333

Abnormalities of the chromosomes 335
Acute leukemias 343
Acute lymphoblastic leukemia 343
Acute myeloblastic leukemia 343
Adult acute leukemia 255
Childhood acute leukemia 253
acute lymphoblastic leukemia 253
Chronic granulocytic leukemia (CML/CGL) 261
Chronic leukemias 261
chronic myeloid leukemia 261
chronic lymphocytic leukemia 261
Chronic lymphocytic leukemia (CLL) 367
clinical staging of CLL 371
Classification 337
Clinical features 343
Course and prognosis 348
differentiation of ALL and AML 346
Disorders with a similar blood picture 347
Disorders with similar clinical features 347
Etiology 333
Fab classification of myelodysplastic syndromes 342
Genetic factors 336
Incidence 343
Laboratory findings 346
Leukemoid reactions 375
myeloid leukemoid reactions 377
myelokathexis 379
Molecular biology of leukemogenesis 333
Myelodysplastic disorders 259
Other chronic lymphoproliferative disorders 374
hairy cell leukemia 374
Prolymphocytic leukemia 375
Radiation 335, 336
Revised fab classification 338
Treatment 348
Viruses 336

Chapter 13: Paraproteinemias 380

Amyloidosis 395
Benign monoclonal gammopathy (benign paraproteinemia) 395
Causes of paraproteinemias 380
Classification of monoclonal gammopathy 380
Heavy chain diseases (HCD) 395
Hyperviscosity syndrome 396
Macroglobulinemia 393
Malignant lymphoma and chronic lymphocytic leukemia 395
Multiple myeloma 383
Principles of the differential diagnosis of monoclonal gammopathies 395
Structure of the immunoglobulins 381

Chapter 14: Lymphoid Tissue Tumors (Malignant Lymphomas) 397

Classification of lymphomas Hodgkin's disease 397
Classification of non-Hodgkin's lymphomas 398
Clinical manifestations in Hodgkin's disease 401
Non-Hodgkin's lymphoma (NHL) 401
Staging 403

Chapter 15: Myeloproliferative Disorders 415

Acute myelofibrosis 440
Causes of polycythemia 415
Familial polycythemia (familial erythrocytosis) 429
Myelofibrosis (myelosclerosis) 431
Polycythemia vera 416
Polycythemia 415
Primary myelofibrosis 432
Pseudopolycythemia 430
Relative polycythemia 430
Thrombocythemia 431

Chapter 16: Platelets, Blood Coagulation, and Hemostasis 441

Acquired hemorrhagic vascular disorders 446
senile purpura (involutional purpura) 446
simple easy bruising 446
Acquired platelet dysfunction 483
Bernard–Soulier syndrome 482
Bleeding disorders 441
Classification of platelet dysfunction 481
Clinical picture 441
Congenital hemorrhagic vascular disorders 453
Eehlers–Danlos disease 455
hereditary hemorrhagic telangiectasia 453
Congenital qualitative platelet defects 481
thrombasthenia (Glanzmann's disease) 481
Defects of platelet enzymes 482
Dysproteinemia 449
Eextravascular factors 444
Functional platelet disorders 480
Granule defects 483
combined defects (αδ-SPD) 483
gray platelet syndrome (δ-SPD) 483
storage pool disease (δ-SPD) 483
Hemorrhagic disorders due to capillary defects: nonthrombocytopenic purpura 445

Hemorrhagic disorders due to platelet abnormalities 455
Hemostatic plug–eventual fate 444
Henoch–Schönlein syndrome (anaphylactoid purpura) 450
autoerythrocyte sensitization 452
mechanical purpura 452
fat embolism 452
orthostatic purpura 452
Idiopathic (hemorrhagic) thrombocythemia 486
Idiopathic cyclical thrombocytopenia purpura 469
Idiopathic thrombocytopenic purpura 459
Immune thrombocytopenia 476
Inherited thrombocytopenias 477
Megakaryocytic hypoplasia 477
Neonatal and inherited thrombocytopenias 476
Normal hemostatic mechanism 441
Pathogenesis 441
normal values 456
physiological variation 457
platelet antigens and antibodies 456
Platelet production 455
Platelet transfusion 478
Secondary thrombocytopenia 470
Symptomatic vascular (nonthrombocytopenic) purpuras 447
Cushing's disease and corticosteroid administration 448
drugs 447
infections 447
scurvy 448
uremia 447
Thrombocytopenia due to drugs and chemicals 472
Thrombocytopenia 457
Thrombocytosis and thrombocythemia 485
Thrombotic thrombocytopenic purpura 475
tourniquet test 457
Types of platelet preparation 478

Chapter 17: Coagulation Disorders 488

Acquired coagulation disorders 519
Anticoagulant drugs 524
Blood coagulation physiology 488
Calcium 490
Clinical disorders due to coagulation abnormalities 503
Congenital coagulation disorders 503
Deficiency of one or more blood coagulation factors 495
Diagnosis of coagulation disorders 496
Disseminated intravascular coagulation 528
Fibrinolysis 495, 496
Foreign surfaces 491
Hemophilia a and hemophilia b 504
Hemorrhage and blood transfusion 532
Hemorrhagic disorders due to circulating inhibitors of coagulation 533
Inhibition of coagulation by acquired inhibitors 495
Inhibitors of coagulation 493
Liver disease 522
Monitoring of anticoagulant therapy 525
Other congenital disorders 518
congenital deficiency of factor II 518
congenital fibrinogen (factor I) deficiency 518
factor XI deficiency 519
factor XII (Hageman factor) deficiency 519
factor XIII (fibrin-stabilizing factor) deficiency 519
inherited dysfibrinogenemia 518
Outline of investigations of a patient with a bleeding tendency 534
Pathogenesis of coagulation abnormalities 495
Plasma fibrinolytic system 494
Plasma protein coagulation factors 489
Plasminogen–plasmin system 494
Platelet and lipid (platelet factor 3) and membrane-binding sites for coagulation factors 491
Theory of blood coagulation 492
Tissue factor (tissue thromboplastin) 491
Treatment of coagulation disorders 499
hematomas and hemarthrosis 503
Vitamin K deficiency 520
von Willebrand's disease 515

Chapter 18: Thrombosis: Clinical Features and Management 538

Anticoagulants 552
heparin 552
low-molecular-weight heparin and heparinoid preparations 554
streptokinase and urokinase 555
thrombosis during pregnancy 557
warfarin or vitamin K antagonists 554
Arterial thrombosis 538
Clinical syndromes of thrombosis 545
Conditions associated with venous thrombosis 541
acquired 542
antithrombin III deficiency 541
dysfibrinogenemia 542
plasminogen abnormalities 542
protein C deficiency 542
Definition of thrombosis 538
Effects of thrombosis 539
Etiology of thrombosis 539
Factors predisposing to both arterial and venous thrombosis 543
Inhibitors of platelet function 551
Investigations of thrombotic disorders 549
Management of thrombotic disorders 550
Microcirculation thrombosis 549
Myocardial ischemia and infarction 545
Occlusive cerebrovascular disease and transient ischemic attacks 546
Peripheral arterial occlusive disease 547
Types of thrombus 538
Vascular disease and arterial thrombosis 539
Venous thrombosis 538
Venous thrombosis 548

Chapter 19: Hypersplenism and Splenomegaly 558

Hypersplenism 559
diagnosis 559
etiology 559
treatment 559
Normal splenic functions 558
Splenomegaly 560
causes 560

effects of splenectomy 562
indications for splenectomy 561
splenic atrophy 563

Chapter 20: Bone Marrow Transplantation 564

Complication of bone marrow transplantation 566
early 566
late 566
Graft versus host disease (GVHD) 567
Indications 564

Chapter 21: Immunodeficiency Disorders 568

Acquired immune deficiency syndrome (AIDS) 569
Assay (ELISA) for the detection of total antibodies to HIV-1 and HIV-2 including HIV-1 group "O" in human serum or plasma 596
Clinical stages of infections with the HIV virus 570
Hematological manifestations of hiv disease 570
Overview of HIV/AIDS 571
Rapid immunoconcentration test for HIV-1 and HIV-2 antibodies flow-through method 593
Third generation double antigen sandwich enzyme linked immuno-sorbent®

Chapter 22: Bone Marrow in Nonhemopoietic Disease 601

Amyloidosis 605
Anorexia nervosa 606
Causes of sea-blue histiocytes 605
Classification of amyloidosis 605
Cystinosis 605
Gaucher's disease 603
Granulomatous disease 602
Kala-azar (visceral leishmaniasis) 603
Mast cell disease 602
Metastatic bone tumors 601
Niemann-Pick disease 604
Osteopetrosis (Albers-Schönberg's disease or marble bone disease) 605
Paget's disease of bone (osteitis deformans) 606
Primary oxaluria 606
Renal osteodystrophy and osteomalacia 606
Sarcoidosis 603
Sea-blue histiocyte syndrome 604
Tuberculosis 602

Chapter 23: Hemoparasites

African trypanosomiasis 626
Babesiosis 638
Bartonellosis 639
Blood flagellates of man 619
Chagas' disease 627
Cutaneous leishmaniasis 624
Hemoparasites 607
malarial parasites 609
mmorphology of malarial parasites stages in thin films 610
Leishmaniasis 622
Plasmid nematodes 631
Wuchereria bancrofti 632
Relapsing fever 639
Trypanosomiasis 625
Tissue roundworms of man 629
Toxoplasmosis 638
Visceral leishmaniasis (kala azar) 623

Chapter 24: Practical Hematology 640

Anticoagulants 642
APTT/PTTK cephaloplastin reagent for partial thromboplastin time (APTT) determination using ellagic acid as activator Liquicecin-e® 703
Automation in coagulation analysis 710
Automation in hematology 668
coulter principle 668
Bleeding time 694
Ivys's method 695
Blood cell counts 649
Blood collection system 644
Blood film examination 664
bone marrow examination and reporting 673
differential cell count 674
examination 673
indications for bone marrow aspiration 676
marrow film preparation 673
Bone marrow examination 672
Capillary fragility test of hess 693
Capillary or peripheral blood 640
Clot retraction 699
Coagulation time 697
Lee and White's method 697
Complete blood count (CBC), blood 654
Cyanmethemoglobin method 646
Dilute blood clot lysis time 707
EDTA 642
Erythrocyte indices 654
mean cell volume (MCV) 654
mean cell hemoglobin (MCH) 654
mean cell hemoglobin concentration (MCHC) 654
Erythrocyte sedimentation rate (ESR) 660
Westergren's method 660
Examination of fetal hemoglobin 683
Factors that play a role in ESR 663
FDPS 708
Fibrinogen degradation products (D-dimer) estimation 720
Hematocrit/packed cell volume (PCV) 648
Hemoglobin (HB) 646
Heparin 643
Interfering factors 663
INR system 717
Laboratory diagnosis of leukemias 687
neutrophil alkaline phosphatase 687
Nonspecific esterase 691
periodic acid–Schiff (PAS) reaction 689
peroxidase 688
sudan black B stain (sheehan and storey) 690
Laboratory diagnosis of vascular bleeding disorders 693
Other methods 647
Platelets 652
Platelets, coagulation and bleeding disorders: laboratory investigations 693
Preparation of a thin blood film 664
Prothrombin time 717
Qualitative assessment of g6pd deficiency 680
Quality control in hematology 691
Quantitative estimation of G6PD 681
Rapid diagnostics 668
RBCS 651
Red cell fragility test 678
Reticulocyte count 677
Sahli's method 646
Sensitive thromboplastin reagent for prothrombin time (PT) determination (ISI=1.0) Uniplastin® 700
Sheard-sanford oxyhemoglobin method 647

Sodium lauryl sulfate method 647
Special anticoagulants 643
Staining of blood films 665
Leishman's stain 666
Giemsa's stain 666
field's stain 666
Simeon's modification of boye's and sterenal's method 667
Tests for sickling 685
Trisodium citrate 643
Venous blood (venipuncture) 640
complications 641
Ways of obtaining blood 640
WBC 649
Wintrobe's method 661
microsedimentation (LANDAU) method 661
Zeta sedimentation rate (ZSR) 662

Chapter 25: Immunohematology Blood Banking 722

ABO grouping 725
ABO testing procedures 724
Antihuman globulin test 730
Biochemistry 723
Blood and its products 752
Blood donors 741
adverse donor reactions 744
donor screening 742
drawing of blood 743
hyperventilation 747
mild, moderate, or severe reactions 745
Blood group antibodies 722
Blood transfusion complications 753
Blood transfusion 741
Choice of material for transfusion 752
Compatibility testing 748
cross-match 748
immediate-spin and thermal incubation modification 749
open-slide method 748
saline cross-match 748
saline tube method 749
Direct antihuman globulin test 737
Genetics of ABO system 723
Indirect antihuman globulin test 740
Investigations in a case of transfusion reaction 754
Laboratory diagnosis of hemolytic disease of the newborn 755
Major applications of DAT in blood group serology 737
hemolytic disease of the newborn (HDN) 737
Naturally occurring antibodies 722
Rh blood group system 727
Rh Du antigen 728
Rh antibodies 729
reagents for Rh(D) grouping 729
Rh(D) grouping procedures 729
slide testing 729
tube method 730
testing for Du 730
SIX-tube method 725
Slide ABO grouping test 725
Subgroups of A and AB 724
subgroups of B 724
antibodies of ABO System 724
anti-H 724
Troubleshooting 755

Chapter 26: COVID and Hematological Changes 766

Nonsevere conditions 766
Severe conditions 667

Chapter 27: Immunoassay: Instrumentation, Automation and Point-of-care Testing 769

BT1500 788
Coralab touch 787
Coralyzer junior 792
Coralyzer smart 790
Councell 23 excel 790
Councell penta 2.0 789
Fluorescence immunoassay 769
point-of-care testing 772
steady-state fluorescence 770
time-resolved fluorescence 771
GR8LAB 786
Hemostar 4CA 795
Hemostar XF 1.0 794
Polymerase chain reaction in clinical diagnosis 775
advantages of PCR 782
applications of PCR 781
point-of-care PCR for clinical diagnosis 782
principle of PCR 776
thermal cycle is PCR 778
uses of PCR 780
Truenat platform 783
Turbodyne SC 793

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